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14. ABSTRACT
The goal of this project was to create a vaccine vehicle utilizing the commensal gut anaerobe, Bacteroides fragilis and the major outer membrane protein, OmpA. We aimed to design a vaccine vehiclethat could quickly and easily be adapted to whatever biodefense agent was considered a threat. As this vehicle could colonize in the human gut, it could stimulate secretory IgA antibodies. A major goal of the work was to design a vector or vectors so that the epitope could be easily changed, and, also, so that the time-limiting step of cloning the modified gene and obtaining the B. fragilis strain with the altered gene would be expedited. Once constructed, manufacture of such a live vaccine could be done very rapidly, since it only involves growing the modified bacteria. We have met these goals and using recent and novel advances in cloning techniques, designed several enhanced vectors to be used in inserting modified genes into a B. fragilis strain lacking the OmpA protein, such that the only OmpA1 protein expressed in the new construct would have the desired epitope displayed. The key research accomplishments were: 1) Construction of ompA deletant strain; 2) Characterization of ompA deletant strain; 3) Transcriptome analysis of WAL186.ompA; 4) Construction of vectors optimized for rapid construction of epitope specific ompAs; 5: Modification of ompA (Insertion of restriction specific sites and insertion of FLAG tag); 6: Construction of ompA reinsertant strain and 7: Construction of FLAG tagged strain

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INTRODUCTION

This study was designed to address a vital concern to our national security: the danger of infectious agents being used vehicles of bioterror. Means to both prevent infection with these agents and to treat infections that may occur are being studied with intensity. Historically, vaccines have been the most efficient method of preventing diseases in large populations, and certainly, in the last decade or so we have seen a precipitous rise in the percentages of multi-antimicrobial resistant bacteria. Over the past 15 years, scientists have developed experimental bacterial vaccine vectors that elicit immune responses against bacterial, viral, protozoan and metazoan pathogens in laboratory animals [28]. Among the advantages of these vaccines is that they are relatively inexpensive to manufacture, they can be given orally, and they can be treated with antibiotics if desired. Ideally, the "delivery vector" should be able to be manipulated to present the antigen optimally. Several organisms have been used in these systems [28] and results suggest that this method is very promising for antigens from both viral and bacterial pathogens to elicit mucosal IgA and T cell responses. If the organism used as the vector can potentially colonize in the host, the potential of eliciting the appropriate response is increased.

Accordingly, the organism that we used as the carrier for the vaccine is *Bacteroides fragilis,* an anaerobic gram-negative bacillus that is a commensal gut bacteria concentrated in the mucosal surfaces. We recently published a comprehensive review of the *Bacteroides* and detailed the properties that render it well suited to adapt to its particular niche [35]. For example, *B. fragilis* has a pronounced capacity to create variable surface antigenicities by multiple DNA inversion systems [12] which gives it an advantage for survival at the mucosal surface (i.e., often the site of attack by host defenses).

The protein chosen for presenting the antigen was the OmpA outer membrane protein, a protein virtually ubiquitous among bacteria and one of the most conserved proteins throughout evolution. OmpA is a protein with many functions and may even assume different conformations depending on external conditions; a recent review referred to the protein as a "molecular Swiss Army Knife" [29]. As such, OmpA is an ideal candidate to serve as the vehicle to carry foreign antigens including those from bioterror agents. The OmpA protein is composed of two domains: one domain forms an eight-stranded transmembrane barrel that has four loops exposed on the outer surface of the bacteria, and one domain links the protein to the peptidoglycan cell wall

layer and serves as an outer membrane anchor. OmpA can have widely varying externally exposed loops that loops be manipulated to present a variety of desired antigenic determinants to the host. OmpAs are able to activate multiple cells in the immune system.

OmpA is one of the major OMPs of *Bacteroides fragilis*, as well. We reasoned that *B. fragilis* with a genetically modified OmpA could colonize the gastrointestinal tract and serve as this live vaccine vector. A wide variety of epitopes could be inserted into the loops, including those from bioterror agents that would be ingested by food or water sources. As a mucosal commensal, *B. fragilis* elicits secretory IgA antibodies which can protect against bioterror organisms acquired by ingestion of food or liquid and also prevent the pathogen or toxin from exiting the GI tract to invade the circulatory system or other organs.

OmpAs from aerobic organisms have been extensively studied for decades by dozens of researchers, and the OmpA like homologs in the gram negative oral pathogenic genus, *Porphyromonas*, have also been recently studied [9,11,15-17,38]. In *P. gingivalis*, two OmpA homologs have been described. Apart from our own work [36], however, the *B. fragilis* OmpA protein has not been characterized at all. As part of this project, **w**e extensively characterized the OmpA outer membrane protein of *B. fragilis.*

The ultimate goal of this project was to create a vaccine vehicle that could quickly and easily be adapted to whatever biodefense agent was considered a threat. As this vehicle could colonize in the human gut, it could stimulate secretory IgA antibodies. A major goal of the work was to design a vector or vectors so that the epitope could be easily changed, and, also, so that the time-limiting step of cloning the modified gene and obtaining the *B. fragilis* strain with the altered gene would be expedited. Once constructed, manufacture of such a live vaccine could be done very rapidly, since it only involves growing the modified bacteria. We have met these goals and using recent and novel advances in cloning techniques, designed several enhanced vectors to be used in inserting modified genes into a B. fragilis strain lacking the OmpA protein, such that the only OmpA1 protein expressed in the new construct would have the desired epitope displayed.

BODY

♦ **Task 1a: Construction of the *ompA1* deletion mutant √ Completed.**

Note: During the course of our studies on *B. fragilis* OmpA, we found multiple homologs for the OmpA gene (the results are discussed further below). To avoid confusion, we are now referring to the major *ompA* gene and protein as *ompA1* and OmpA1, respectively.

METHODOLOGY: Construction of WAL186Δ*ompA* (*ompA* deletion mutant). An in-frame deletion of *ompA* was constructed by a two-step double cross-over technique with the pYT102 suicide vector [1]. Briefly, 800 bp fragments of the upstream and downstream regions (including approximately 50-100 bp of the beginning and end of the gene) of *ompA1* were amplified using specific primers to which appropriate restriction sites were added for subsequent cloning into the suicide vector (Table 1). pYT102 was digested with BamH1 and HindIII and gel purified. PCR amplicons were digested with BamH1/NCO1, or HindIII/NCO, respectively, and mixed with BamH1/HindIII digested pYT102 in a three part ligation as described [25].

Chemically competent *E. coli* DH5α was transformed with pYT102::'*ompAupdown*' and transformants selected by chloramphenicol. pYT102::'*ompAupdown*' was mobilized into *B. fragilis ADB77* in a three part mating with *E. coli* DH5α/ pYT102::'*ompAupdown*' and *E. coli* HB101/pRK231 [1]. Cointegrants were selected by gentamicin (50 µg/ml), rifampicin (50 µg/ml) and tetracycline (2 µg/ml), confirmed by colony PCR using primers designed to detect the recombinant junction and maintained on media with tetracycline. The second step recombination was done as described [1]. Trimethoprim resistant colonies were screened to confirm that they were tetracycline sensitive, and further screened by PCR with sets of both internal and junction primers to confirm that they were the desired deletion resolution products. The *ompA1* deletant was named WAL186Δ*ompA*.

RESULTS: Construction and characterization of WAL186Δ*ompA1*. Sequence analysis confirmed the deletion of the *ompA1* gene and SDS PAGE analysis confirmed the lack of the OmpA1 protein in WAL186Δ*ompA1* (Figure 2). No other OmpAs were detected in WAL186 WAL186Δ*ompA1* using silver stain analysis (data not shown). We believe that although *ompAs* 2, 3, and 4 are transcribed in WAL108 (and *ompAs* 2 and 3 in WAL186Δ*ompA1*), OmpAs 2, 3 and 4 are not expressed in sufficient levels to be detected in the SDS-PAGE analysis of the outer membrane preparation.

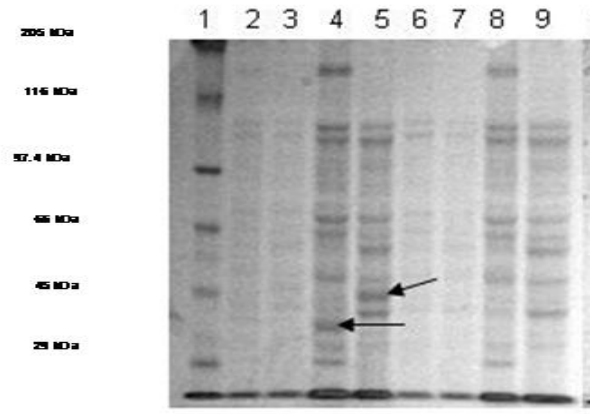


Figure 1: **SDS-PAGE analysis of OmpA parental and deletant strains.** Lane 1: Molecular weight (MW) markers 205, 116, 94.7, 66, 4 and 29 kDa, respectively; 2 and 3: *B. fragilis* WAL 108 wild type cell lysates; 4 and 5: WAL 108 wild type Triton pellet (25'C and boiled, respectively); 6 and 7: WAL 186 Δ*ompA* cell lysates; 8 and 9: WAL 186Δ*ompA* Triton pellet (25'C and boiled, respectively)

Task 1b: Characterize WAL186Δ*ompA1* √ **Completed.**

RESULTS: Colony characteristics. Colonies of WAL186Δ*ompA1* were much smaller than those of WAL108 (parental) or WAL360+*ompA*1; these results echoed those seen with WAL67*ΩompA1*.

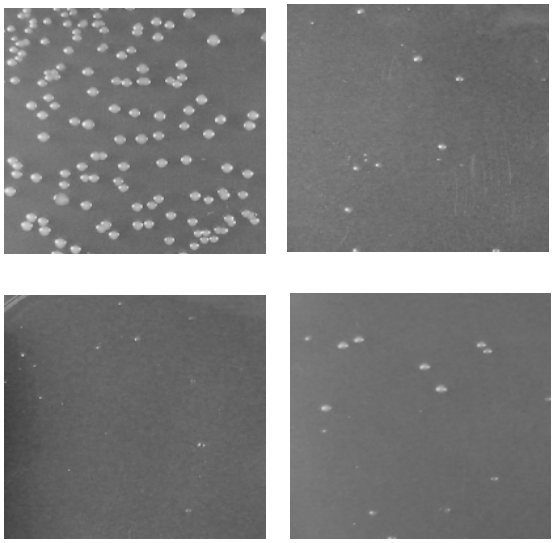


Figure 2. Colony size of *B. fragilis* strains. Overnight cell cultures were diluted 1/100000 in dilution blank and plated on BHI-agar supplemented with thymine and incubated anaerobically for 2 days at 37 'C. Upper left--WAL26, Upper right-- WAL 108, Lower left--WAL 186 and lower right--WAL 360.

***B. fragilis* OmpA1 does not appear to be important in transport of antimicrobials into the cells.** Using the spiral gradient endpoint (SGE) technique {Wexler, 1996 3586 /id;Wexler, 1991 2294 /id, we measured MICs for a wide variety of antimicrobials including β-lactams (ampicillin, cefoperazone, cefoxitin, cephalexin, ceftizoxime), carbapenems (doripenem, ertapenem, faropenem, imipenem, meropenem), quinolones (ciprofloxacin, gatifloxacin, norfloxacin, levofloxacin, moxifloxacin), chloramphenicol, metronidazole, clindamycin, erythromycin and tetracycline. No significant change was seen in MICs for WAL 108 and WAL186Δ*ompA1*.

Resistance of WAL108 and WAL186Δ*ompA1* to osmotic stress. Challenge with SDS, acid, and high salt were performed as described by Wang for *E. coli* OmpA1 [34] using media and incubation conditions appropriate for *B. fragilis*. WAL 186Δ*ompA1* was more sensitive than WAL 108 to exposure to both SDS and high salt. Exposure of WAL108 to 5M NaCl for 2 hours resulted in a 3 $\log_{10}$ reduction in growth ($1x10^8$ to $5x10^5$); WAL186Δ*ompA1* did not grow at all after exposure to high salt. Similarly, growth of WAL108 on media containing 0.05-0.2% SDS resulted in a 3 $\log_{10}$ reduction in growth as compared to growth on media without SDS ($1x10^8$ to 5.3, 5, and $4.5x10^5$ on 0.05%, 0.1% and 0.2% SDS, respectively); WAL186Δ*ompA1* did not grow at all on media containing even 0.05% SDS. No change in growth between WAL108 and WAL186Δ*ompA1* was seen after exposure to low pH.

Response of *ompA* transcription levels to high salt. Exposure of WAL 108 and WAL186Δ*ompA1* to 200mM NaCl significantly reduces transcription of all four *ompA* homologs in WAL108 and of *ompAs 2, 3*, and *4* in WAL186Δ*ompA1* (Table 2). Gram stain analysis indicated similar morphology in both WAL108 and WAL186Δ*ompA1* grown on normal media (somewhat pleomorphic gram-negative rods). Gram stain analysis of the strains grown overnight with 200 mM NaCl added revealed that both WAL108 and WAL186Δ*ompA1* assumed very small, round forms.

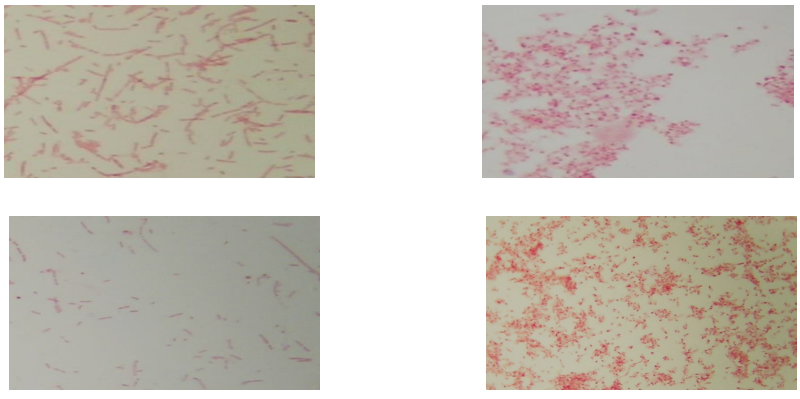


Figure 3: Upper and lower left panels: typical long rod of *B. fragilis*, both in the parental and in the deletant strain on the left. Upper and lower right panels: in the presence of 200 mM NaCl, only small round forms are seen.

***B. fragilis* WAL186Δ*ompA1* is more sensitive to oxygen than WAL 108.** The oxygen sensitivity of the *ompA1* deletant was measured in an agar tube assay as described [26]. Strains were grown in BHIS/thy anaerobically at 37°C. One hundred microliters of overnight (stationary phase) cultures were mixed with 5 ml of BHIS/thy and 0.4% agar in a clear polystyrene tube and incubated aerobically at 37°C for two days. The distance between the top of the agar and the visible growth within the agar was measured. WAL186Δ*ompA1* was more sensitive to oxygen stress than either WAL108 or WAL360+*ompA1* (the *ompA1* reinsertant), indicating that the absence of the *ompA1* gene, and not some downstream effect or other random mutation, was responsible for the change in sensitivity to oxygen (Figure 3). The average measurements between the top of the agar and the visible growth within the agar were: *B. fragilis* 638R, 6.8 mm; WAL108, 9.2 mm; WAL186Δ*ompA1*, 14 mm; and WAL360+*ompA1*, 9.8 mm. Incubation for an additional 24 hours did not affect the results.

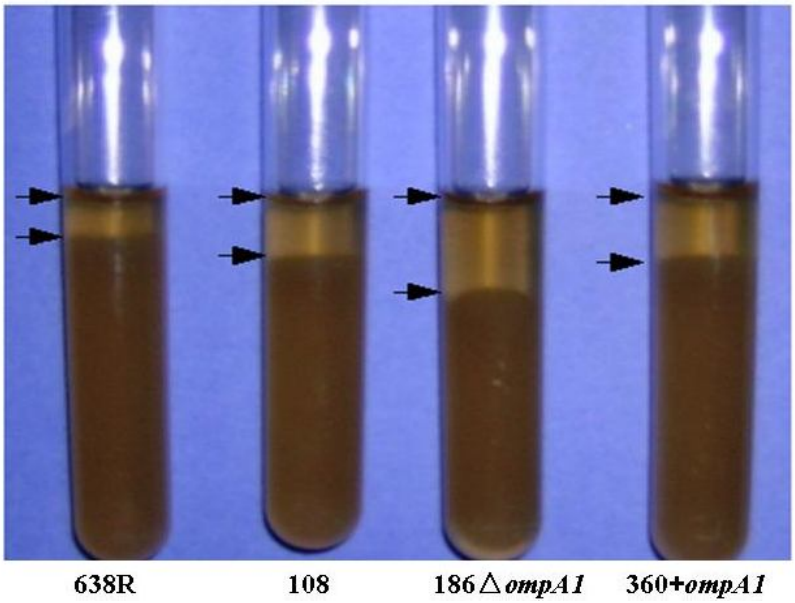


Figure 4: Effects of oxygen stress on *B. fragilis* (108, parental; 186-ompA deletant, 360- ompA reinsertant

Identification of *ompA* homologs in *B. fragilis*. In studies completed before the publication of the *B. fragilis* genome sequence, we identified three additional *ompA* genes using a TBLASTN search against genomic data from the *B. fragilis* sequence data

(http://www.sanger.ac.uk/Projects/B_fragilis/). The amino acid sequences for OmpA1, OmpA2 and OmpA4 are identical for *B. fragilis* strains ATCC 25285 and *B. fragilis* 638R. There are two amino acid differences between *B. fragilis* 25285 OmpA3 and *B. fragilis* 638R OmpA3 (F19 □L, K225 □R). According to our model, F19 is in the leader sequence, before the cleavage site and K225 is in the periplasmic portion, just after the last β-strand, thus the barrel portion of the four OmpA homologs is completely conserved in these two strains. Subsequent analysis of the annotated *B. fragilis* sequence revealed three additional *ompA* family homologs (OmpAs 5, 6 and 7) that are somewhat removed from the OmpA1-4 cluster but do contain the OmpA signature domain at the C-terminal (http://expasy.org/prosite/PDOC00819). OmpA5 is approximately the same lengths as OmpAs1-4 (372-399 amino acids); OmpA6 has 224 amino acids and OmpA7 has 616 amino acids. OmpAs 1-7 correspond to *B. fragilis* ATCC 25285 genes BF 3810, 1988, 1689, 1959, 3801 and 1285, respectively. Alignment of the homologs was helpful in the prediction of positions of β-sheets and loops, below.

Nucleotide sequences and genetic organization of *B. fragilis ompAs*. Regions upstream of the start codon for *B. fragilis ompAs* were examined and potential promoters identified based on the consensus promoter sequences described by Bayley et al [2] (Figure 4c). All four genes had regions conforming to possible promoters for *B. fragilis* genes. *ompA2* and *ompA4*, which share the most homology of the four genes, are separated by ~10000 bp, are in inverse orientation and may be the result of a duplication event. Both genes have very conserved upstream sequences that have low level homology (Expect level e^{-5}) to *Vibrio cholera otnG* (which is involved in cell wall polysaccharide biosynthesis.) Also, pairwise BLASTN of the upstream sequences revealed a highly conserved 200 bp region (Expect level $-5e^{-50}$) upstream of the *otn G* -like sequences.

Amino acid sequences and signal peptide sequence of *B. fragilis* OmpAs. The homology of OmpAs 1-4 extends throughout the entire ORF with 30% to 34% identity and 49% to 50% similarity at the primary amino acid level. OmpA2 and OmpA4 are the most homologous pair with 84% identity and 89% similarity at the amino acid level. The homology among all of the OmpA homologs of *B. fragilis* is more substantial in the carboxy-terminal end of the protein and all have significant and comparable homology with the conserved domain database entry for the OmpA family (data not shown). Both in *E. coli* OmpA and *Pseudomonas* OprF, the N-terminal transmembrane domain and the C-terminal periplasmic region are connected by a hinge region [7] composed of an alanine-proline (A-P) repeat preceded by a

phenylalanine a few residues earlier. The last membrane-spanning segment of OmpA [33] does exhibit the C-terminal motif (including a terminal phenylalanine considered essential in many OMPs [31]), as does *P. aeruginosa* OprF [37]. While no A-P repeat was seen in *B. fragilis* OmpA, there is an arginine-proline-methionine-proline (RPMP) segment, preceded by two phenylalanines (2 and 6 bases earlier). This segment may serve the same function as the OmpA hinge. When we examined the sequence just before the potential RPMP hinge in the *B. fragilis* OmpAs 1-4s, we found striking similarity to the corresponding regions in *E. coli* and *Shigella* OmpAs (Figure 4b). *B. fragilis* OmpAs 1-4 sequences have a "terminal" phenylalanine as the last amino acid of the last β-sheet of the β-barrel, which is consistent with OmpAs from other species. In addition, OmpAs1-4 homologs have hydrophobic amino acids at positions -3, -5, -7 and –9 relative to the terminal phenylalanine that is also characteristic of porin proteins. The corresponding region in *Pseudomonas* is different, although completely conserved in four different species. Alignment of this region of *B. fragilis* OmpA1-4 with the last β-sheet of *E. coli* OmpA was helpful in constructing the alignments used in the structural predictions for *B. fragilis* OmpA1 (below). The carboxy-terminal 17 to 27 amino acids of the *B.fragilis* OmpA1 homologs do not align with the OmpA-domain consensus and may reflect the phylogenetic distance of *B. fragilis* from the constituents that define the OmpA family.

Figure 5. Putative alignment of the eighth ß strand (region preceding hinge in *E. coli* OmpA and possible hinge (RPMP) in *B. fragilis* OmpA)

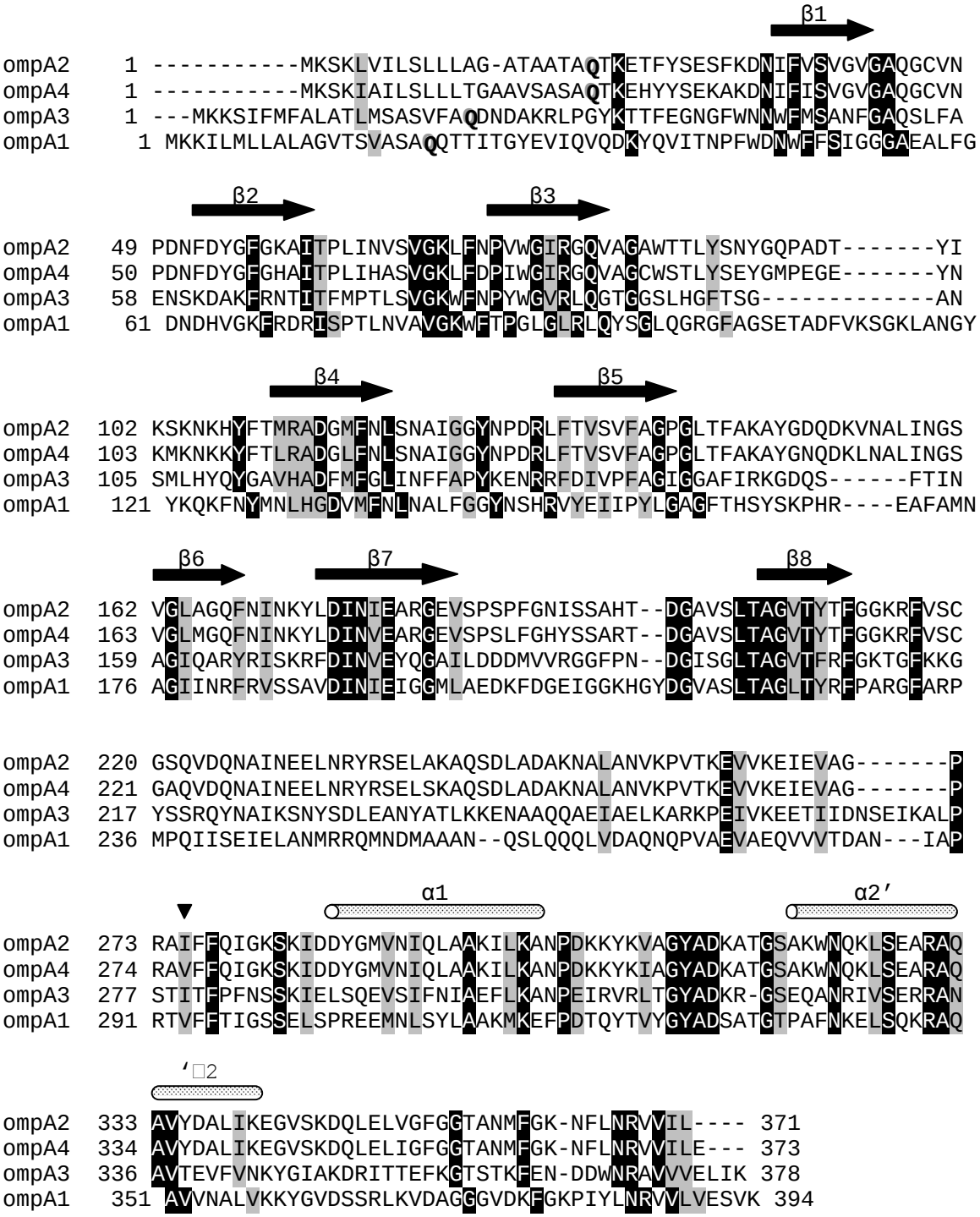
```
B. fragilis OmpA1          1 GYDGVASLTAGLTYRF
B. fragilis OmpA2          1 --DGAVSLTAGVTYTF
B. fragilisOmpA3           1 --DGISGLTAGVTFRF
B. fragilisOmpA4           1 --DGAVSLTAGVTYTF

E coli OmpA                1 PDNGMLSL--GVSYRF
Shigella OmpA              1 PDNGLLSL--GVSYRF

P. aeruginosa OprF         1 SGEWMAGL--GVGFNF
P. fluorescens OprF        1 KWDYSALV--GLGVNF
P. tolaasii OprF           1 KWDYSALV--GLGVNF
P. marginalis OprF         1 KWDYSALV--GLGVNF
```

–Note "terminal" phenylalanine and hydrophobic amino acids at -1, -3, -5, -7, and –9

Figure 6: Alignment of deduced amino acid sequences for *B. fragilis* OmpA, OmpA1, OmpA3 and OmpA4. The sequences were aligned with CLUSTALW and presented with BOXSHADE. The NH_2-terminal glutamine that remains after the signal peptide cleavage is indicated by a **Q**. Positions where amino acid identity is shared by all four homologs are highlighted in black. Positions where amino acid similarity is shared by all four homologs are highlighted in gray. Black arrows indicate the eight predicted β-strands of the membrane-spanning β-barrel. The black triangle indicates the beginning of the OmpA domain. The stippled tubes indicate alpha-helices predicted by 3D-PSSM.



β-sheet prediction and model prediction. The prediction of β-sheets was kindly done by Dr. Tilman Schirmer (University of Basil) according to his published method [27].

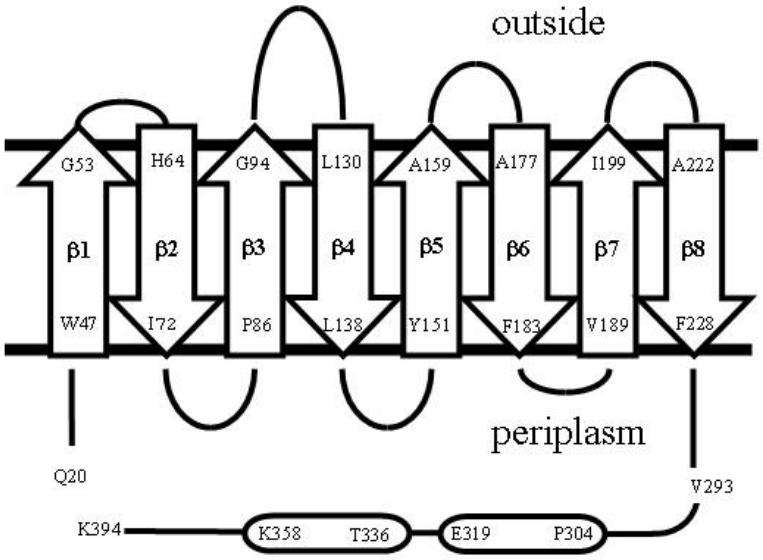


Figure 7. Schematic diagram of the proposed trans-membrane fold. A two-dimensional representation of the 8-strand β-barrel of *B. fragilis* OmpA1 spanning the outer membrane based on a ClustalW alignment of the sequences of the four *B. fragilis* OmpAs, *E. coli* OmpA, and *P. aeruginosa* OprF. The amino-terminal residue after signal cleavage is Q20 and the carboxy-terminal residue remains K394. β-strands are labeled and are represented by arrows with the first and last predicted amino acid indicated. Loops are indicated by arcs between adjacent β-barrels. Elongated circles represent predicted α helices. The OmpA-domain is located in the periplasm and starts at position V293.

The multiple alignment based on ClustalW was threaded onto the *E. coli* OmpA crystal structure and regions of conservation analyzed by surface-mapping of phylogenetic information using the program CONSURF [8,13] and visualized with the Protein Explorer program (http://www.umass.edu/microbio/chime/pe_beta/pe/protexpl/).

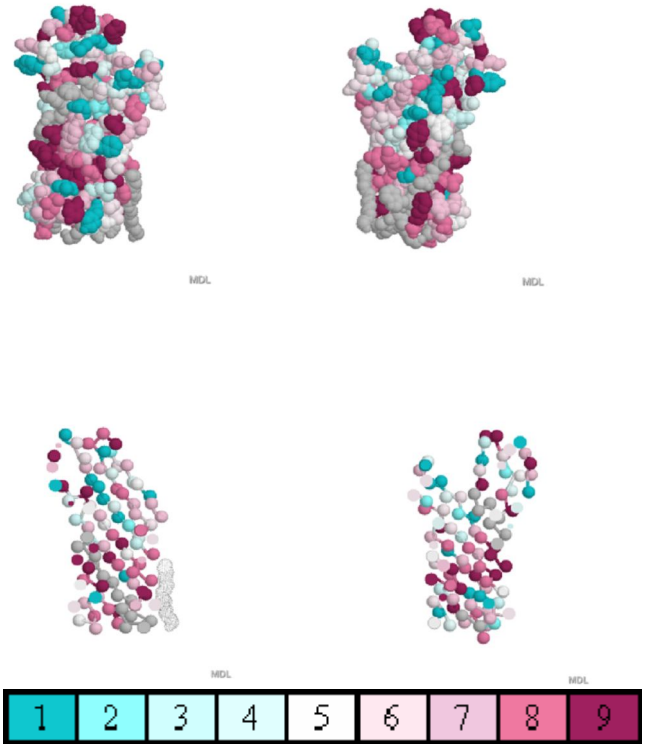


Figure 8: Slab Mode showing *B. fragilis* OmpA barrel threaded onto the *E. coli* OmpA crystal structure. Upper panel: outside surface of barrel. Lower panel: Inside surface of barrel (Beta carbons (alpha for Gly) are space-filled. Conservation is color-keyed as indicated (blue=least conserved to maroon=most conserved.)

Transcription of *ompA* homologs in *B. fragilis* 638R, WAL108, WAL67 Ω*ompA1* , and WAL186Δ*ompA1*.

METHODOLOGY: Quantification of gene expression by quantitative comparative real time RT-PCR. Our previous studies had confirmed the presence of several homologs of the ompA gene in the *B. fragilis* genome and RT-PCR demonstrated that all of the genes were transcribed (data not shown). We therefore wanted to measure the relative expression of the four homologs most closely resembling *ompA1*. RNA was extracted as described in several of our publications [22,23,25]. Two-step real-time PCR was performed with the Cepheid SmartCycler® using the Quantitect® SYBR® Green one-step RT-PCR kit (Qiagen). Primers were designed to amplify products between 130-170 bp in size and were added to the reactions at a final concentration of 1.0 µM each. RNA samples were added to the reactions to result in 200 ng/reaction, except for the 16SRNA samples that were added to a final amount of 200 pg/reaction. Expression levels were measured as an amount of cDNA as extrapolated by a cycle

threshold (Ct) value from the standard real time PCR growth curve. The Ct was the cycle number at which the growth curve attained exponential growth and was thus the highest concentration of template. In order to rule out any non-specific products resulting from primer dimers, melting curve analysis of the amplified products was performed. RNA expression was normalized to the parental strain by using 16SRNA. Expression results were quantified by the comparative cycle threshold approximation method [30], using the assumption that the PCR growth curve efficiency for all reactions is 100% and that the DNA concentration doubled at each cycle:

ΔΔCt (fold-change in expression) = 2(Ctparental - Ct16srRNA) – 2(Ctdeletant-Ct16srRNA). Data were analyzed by Student's T test and a value of $P < 0.05$ was considered significant. A ≥2-fold change in expression compared to the parental strain was considered significant.

RESULTS: Transcription levels of the *ompA* homologs in the *B. fragilis* constructs as measured by quantitative RT PCR are shown in Table 2. The major transcribed homolog is *ompA1*, followed by *ompA3*, *ompA2* and *ompA4*, respectively. Our studies with WAL67Ω*ompA1* had already indicated that *B. fragilis* OmpA1 is important in maintaining cell structure; therefore, we initially assumed that the organism might compensate for the loss of *ompA1* by increasing transcription of one of the other *ompA* homologs. However, we found that transcription of *ompA4* is significantly reduced in WAL186Δ*ompA1*, suggesting the presence of a positive regulatory mechanism to control *ompA4* transcription that is dependant on *ompA1*. Interestingly, the same effect is not seen in WAL67*ΩompA*. We speculated that perhaps the truncated *ompA1* gene or gene product in the disruption mutant can fulfill the function of the full length product in regulating *ompA4* transcription.

Table 1: Comparative transcription levels of *ompAs* 1-4

	Strain and expression Ct				
gene	**108**	**186**	**67**	**108/ NaCl**	**186/NaCl**
16s rRNA	22.33	23.17	22.07	21.42	21.16
ompA1	20.21	**0**	22.69	**27.62**	0
ompA2	27.76	27.9	27.25	**36.52**	**38.88**
ompA3	25.44	26.74	25.14	**37.21**	**39.54**
ompA4	30.94	**37.59**	31.89	**0**	**0**

Characterization of the transcriptome of WAL186ΔompA1 compared to parental strain and to *B. fragilis* strain 638R. In order to better describe the function of *B. fragilis* OmpA, we conducted a comparative transcriptome analysis of the strains to obtain information about any additional changes that might occur as a result of this deletion. We were surprised to find significant changes in almost one quarter of the transcriptome, encompassing all categories of gene products. Microarray data yields a tremendous amount of information, and it is challenging to discern meaningful patterns. Currently, our model is consistent with the hypothesis that the deletion of the OmpA1 protein compromises cellular integrity and results in a global stress response. We are currently submitting a description of the changes in the transcriptome, and submitting the transcriptome data to the Gene Expression Omnibus at NCBI, but will distill very brief highlights into this report.

Preparation of cDNA and transcriptome analysis. Strains were grown to midlog phase and RNA extracted with the Qiagen RNAeasy kit using the RNAProtect protocol. RNA was treated with TURBO-RNAase free DNAase (Ambion) as specified in Nimblegen protocols. RNA purity was assessed by $OD_{260/280}$ ratios and RNA integrity was assessed on a FlashGel system (Lonza Group, Baltimore, MD). Double stranded cDNA was produced using the SuperScript Double-Stranded cDNA synthesis Kit (Invitrogen).

Microarray. cDNA was submitted to Roche Nimblegen, Inc. (Madison, WI) for analysis on a gene-centric microarray. The probes corresponded to genes of *B. fragilis* NCTC 9343, an abdominal isolate that has been designated as the *B. fragilis* type strain and sequenced at the Wellcome Trust Sanger Institute [6]. Labeling and hybridization of the microarray were performed by Nimblegen personnel. The cDNA was labeled with Cy-3; the array format was 385,000 individual 60 mer probes, with 6-20 probes per target and 1-4 replicates per probe. The scanned images were imported into NimbleScan software (Nimblegen) and the data was extracted and pair reports created.

Analysis of microarray data. The raw data was normalized by quantile normalization [4] and gene calls generated with the RMA algorithm [10]. Array Star software (DNAstar) was used to analyze the data. P- and F-values are calculated by the F-test ANOVA method using the False Discovery Rate (FDR) multiple testing correction method [3]. The genes with altered expression levels were subjected to cluster analysis using the hierarchical method. We added several annotation data bases to that provided by Nimblegen, including gene identification, gene ontology biological process and function, COG function and used the added annotations to

discern patterns and describe the family(ies) of gene functions affected. Table 2 shows the transcription levels of the various ompA homologs. The other *ompA* homologs did not show significant changes in transcription levels when the ompA1 gene was deleted, which is consistent with the lack of an observed OmpA protein in SDS PAGE gels of WAL186Δ*ompA1*.

Table 2: Expression levels of *ompA* homologs in microarray

								Expression level (ln2)			
GENE	**GENE #**	**Fold change 108 to 186**		**Fold change 186 to 360**		**Fold change 108 to 360**		**WAL 26**	**WAL 108**	**WAL 186**	**Strain 360**
OmpA	BF3810	13.6	down	18.7	up	1.4	up	15.4	14.6	10.8	15.1
OmpA2	BF1689	1.1	down	1.2	down	1.3	down	12.9	13.1	12.9	12.7
OmpA3	BF1285	1.8	up	4.2	down	2.3	down	12	12.7	13.6	11.5
OmpA4	BF1681	1.5	up	1.6	down	1.1	down	6.1	5.9	6.5	5.8
OmpA5	BF1988	2.5	down	1.3	down	3.2	down	10.3	11	9.7	9.4
OmpA6	BF1959	1	up	1.1	up	1.1	up	14.2	12.5	12.5	12.6
OmpA7	BF3801	2.2	down	1.2	up	1.9	down	11.8	12.2	11	11.2

General categories of genes most affected by the deletion of *ompA*. Figure 5 shows the categories of major genes with high transcription values ($>\ln_2 12$) in either the parental or deletant strain that showed a $\geq$4-fold difference in transcription values when *ompA1* was deleted. Genes were subdivided into COG categories, and the expression values were totaled within the COG (**C**lusters of **O**rthologous **G**roups) category (as a rough estimate of total activity of the particular category); these numbers are indicated as bars above the x-axis. Numbers of genes up or downregulated within the COG categories are indicated as bars below the x-axis. The COG categories with the highest total transcription upregulation in WAL 186 Δ*ompA1* (i.e., total expression and proportions of genes upregulated) included transcription factors (COG K), genes involved in signal transduction (COG T) and genes involved in host defense, including efflux genes (COG V). COG categories with significantly reduced transcription levels and with increased numbers of genes with downregulated transcription included genes involved in energy production and conversion (COG C) and in cell wall, membrane and envelope biogenesis (COG M).

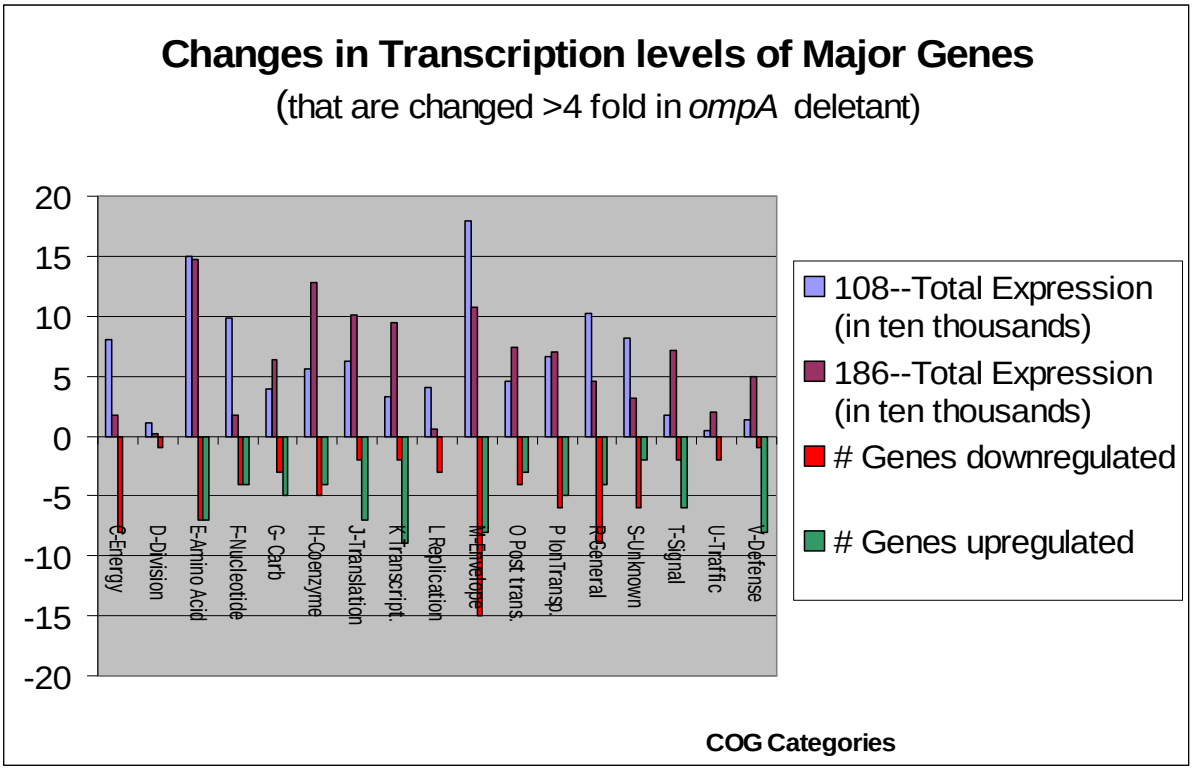


Figure 9: **Changes in transcription levels of major genes.** Genes with transcription levels (ln2) of >12 in either WAL 108 or WAL 186 Δ*ompA*, and in which there was at least a four fold change in the other strain, were analyzed by COG category.

Task 1c: Compare parental and *ompA1* deletant strains in a rodent abscess virulence model. **√ Completed.**

Rat intra-abdominal sepsis model. Animal experiments were performed by Dr. Andrew Onderdonk at the Channing Laboratory, Brigham and Womens's Hospital, Harvard Medical School (Boston, MA). Male VAF Wistar rats (175–200 g) purchased from Charles River Laboratories were used for all in vivo experiments. Rats were housed according to Brigham and Women's Hospital and Harvard Medical School guidelines for care and use of animals and received sterile water and food *ad libitum*. Surgery was performed as described [18,19]. Rats were anesthetized with a single intraperitoneal (ip) injection of sodium pentobarbital (7.5 mg/rat; Abbott Laboratories), the abdomen was shaved and cleaned with iodine, and a 0.5-cm incision was made through the anterior abdominal wall. After 0.5 mL of inoculum was placed in the abdominal cavity, the incision was closed with silk suture. Mortality within 4 h of surgical implantation of the inoculum was considered to be an anesthetic-related death, and rats that died in that way were not included in the final results for each experiment. The inoculum in

peptone yeast glucose broth containing 10% (vol/vol) sterile cecal contents was used for experiments in which mortality was the end point. After surgery, rats were observed twice per day until the completion of the experiment. Both the parental and the OMP A deletion mutant yielded abscesses in the majority of animals at 1 x 10-8, -7, -6 and -5 cfu/animal.

Task 1d: Compare parental and *ompA1* deletant strains in invasion assays of brain microvascular endothelial cells (BMEC) and macrophages. **√ Completed (model adjusted).** This assay was done in the laboratory of Dr. Prasadarao Nemani (University of Southern California) [20,21]. Briefly, bacteria were added to confluent monolayers of BMEC and incubated for ~1.5 hours at 37 ^{o}C. The monolayers were washed and the number of cell associated bacteria determined after the BMEC were lysed with 0.5% Triton x. The number of intracellular bacteria was determined after extracellular bacteria were eliminated by incubation of the monolayer with antibiotic. The released intracellular bacteria were plated on blood agar and then counted. Additionally, the entry and intracellular expression of *B. fragilis* in macrophages was measured [32]. In these initial studies, we determined that *B. fragilis* could survive the incubation in the CO_2 atmosphere used for these studies, but we did not observe any binding or invasion of the bacteria. Therefore, we were not able to demonstrate invasion of *B. fragilis* into BMEC. The assay is not performed in an anaerobic chamber, and while *B. fragilis* can survive for lengthy periods in air, it is likely that the *B. fragilis* needs to be metabolically active to be able to invade the BMEC (and metabolism will only occur in anaerobic conditions).

We set up an alternate assay to look at adherence to intestinal epithelial cells as well as an assay for biofilm formation We found that both the OmpA1 deletant (WAL 186) and the parental strain (WAL 108) were deficient in 1) adherence to intestinal epithelial cells [24] and

Strains	OD550			SD
WAL12	0.26	0.23	0.245	0.021213
WAL26	0.11	0.094	0.102	0.011314
WAL108	0.006	0.012	0.009	0.004243
WAL186	0.01	0.017	0.0135	0.00495

	Mean	SD
WAL12	0.245	0.0212
WAL26	0.102	0.0113
WAL108	0.009	0.0042
WAL186	0.0135	0.0049

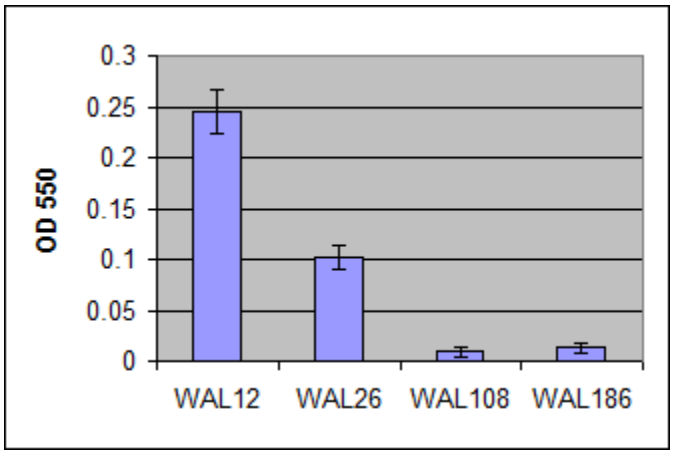


Figure 10: Absorbance reading of redissolved Crystal violet used to stain the attached cells.

2) biofilm formation compared to strain 638R (WAL 108 is a *thy*⁻ deficient mutant of 638R, engineered to be more amenable to use in allelic exchange experiments). We had not expected that a *thy* mutation would have this effect. In order to more closely examine the differences that the *thy* disruption is creating in WAL108 (since this will be the basic strain for our vaccine vector, we are doing transcriptome analysis on these strains as well.

♦**Task 2: Determine positions of external loops of OmpA**

Task 2a: Tag proposed external loops of OmpA. √ Completed.

Task 2b. Express FLAG-tagged OmpA in *E. coli.*

Task 2c. Express FLAG-tagged OmpA in *B. fragilis*.

We successfully cloned the *ompA1* gene into the pet27b vector in a non-expressing strain. However, this needed to be subcloned into an expressing strain in order to express and properly export the protein to the outer membrane. We repeatedly tried various permutations in order to express the *ompA* gene in *E. coli*, but found that the protein expression was toxic to the cells. We were able to obtain constructs in which the *ompA1* gene was expressed but these clones were unstable and could not be reproducibly propagated. These experiments have "morphed" into Task 4, and we are using allelic exchange to insert the modified *ompA1* genes directly into the chromosome of the *ompA1* deletant of *B. fragilis*. Thus, the modified gene will be in exactly the position as the original gene, and will be under the same regulatory controls as the original gene.

♦**Task 3: Construct genetically modified OmpAs with specific insertions or deletions in outside loops**

Task 3a. Construct OmpAs with specific deletions in outside loops.

The purpose of Task 3a was to identify those portions of the loops that were responsible for the virulence in the rat abscess model (Task 1C). Since there was no difference in virulence between the parental strain (WAL 108) and the *ompA1* deletant (186) in the model (see Task 1c), there was no need to proceed with Task 3a. (This possibility was anticipated in the original proposal and the budget actually assumed that this task would not need to be done).

Task 3b. Construct OmpAs with specific epitopes inserted into outside loops.

Completed. √

Construction of genetically modified OmpAs with added restriction sites. We designed a plasmid so that epitopes could be easily changed when desired. Since we needed restriction sites that would cut specifically around the loops and nowhere else on the plasmid, we had to identify restriction sites that were not present on either the plasmid or the *ompA1* gene, and then introduce those sites into the *ompA1* gene. Two restriction sites, AatII and SalI, were identified that were not present in *ompA*, but unfortunately there was one AatII site in the plasmid. To eliminate this AatII site, the plasmid was digested with AatII, blunted with Shrimp Alkaline Phosphatase and incubated with ligase to promote self-closing of plasmid at the blunt ends. Elimination of the AatII site was confirmed by restriction digest analysis of the modified pADB242b; the modified plasmid was not digested by AatII and was named pADB242c.

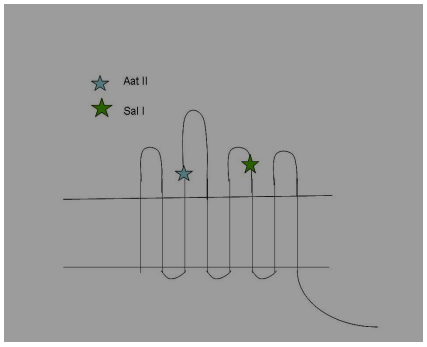


Figure 11: AatII and Sal1 sites on loops 2 and 3 of OmpA, respectively

Note: for the remainder of this report, the modifications done to the suicide vector to make it suitable for rapid cloning were done with both pADB242b and pADB242c; the figures are mainly labeled with only pAD242b for simplicity.

The AatII site was introduced into the *ompA1* by PCR amplification using B. fragilis WAL 108 genomic DNA as template. Two separate PCR fragments were amplified, one from the upstream portion of *ompA1* to loop 2 (with a HindIII site at the 5' end of the forward primer and with a with the AatII site included in the reverse primer) and from loop 2 region (with the AatII site included in the forward primer and a BamH1 site at the 5' end of the reverse primer) to

the downstream portion of *ompA*. The two PCR fragments were digested with BamH1 and HindIII, respectively, and then assembled in BamH1/HindIII digested pBR322 in a 3 part ligation and transformed into *E. coli* Top 10.

The SalI site was introduced by performing another round of PCR amplification using pBR322::OmpA::AatII as template. As described above, two PCR fragments were amplified , one from the upstream portion of ompA1 to loop 3 (with a HindIII site at the 5' end of the forward primer and the SalI site included in the reverse primer) and from loop 3 region (with the SalI site included in the forward primer and a BamH1 site at the 5' end of the reverse primer) to the downstream portion of ompA. The two PCR fragments were digested with BamH1 and HindIII, respectively, and then assembled in BamH1/HindIII digested pBR322 in a 3 part ligation and transformed into E. coli Top 10. The resultant plasmid was pBR322::ompA/AatII(loop 2)SalI (loop 3).

The modified ompA/AatII(loop 2)SalI (loop 3) was inserted into pADB242c by the ligation independent cloning approach described below. Thus, we have ompA/AatII(loop 2)SalI (loop 3) in both pBR322 and pADB242c backbones.

Construction of *ompA1* fragment with AatII-FLAG sites inserted. PCR was used to amplify two separate fragments using *B. fragilis* WAL 108 genomic DNA as template. One fragment was amplified using a forward primer ~800 bp upstream of ompA1 with a hindIII site at the 5' end, and a reverse primer at the loop2 site with an AAtII site on the 5' end. The other amplicon was made using a forward primer starting at loop 2 with an AAtII-FLAGG sequence at the 5' site of the forward primer, and a reverse primer ~800 bp downstream of the end of the *ompA1* gene, with a BamH1 sequence at the end. The first and second fragments were assembled by digesting with HindIII+AatII and with AatII+BamHI, respectively, cloned using BamH1/HindII digested pBR322 and transformed into *E. coli* DH5á. The *ompA1-AatIIFLAG* sequence was then amplified using pBR322::*ompA1*aatii/FLAG as template and the LIC adapted primers as forward and reverse primers, and then cloned into the LIC adapted pADB242c (described below) with a ligation independent cloning protocol.

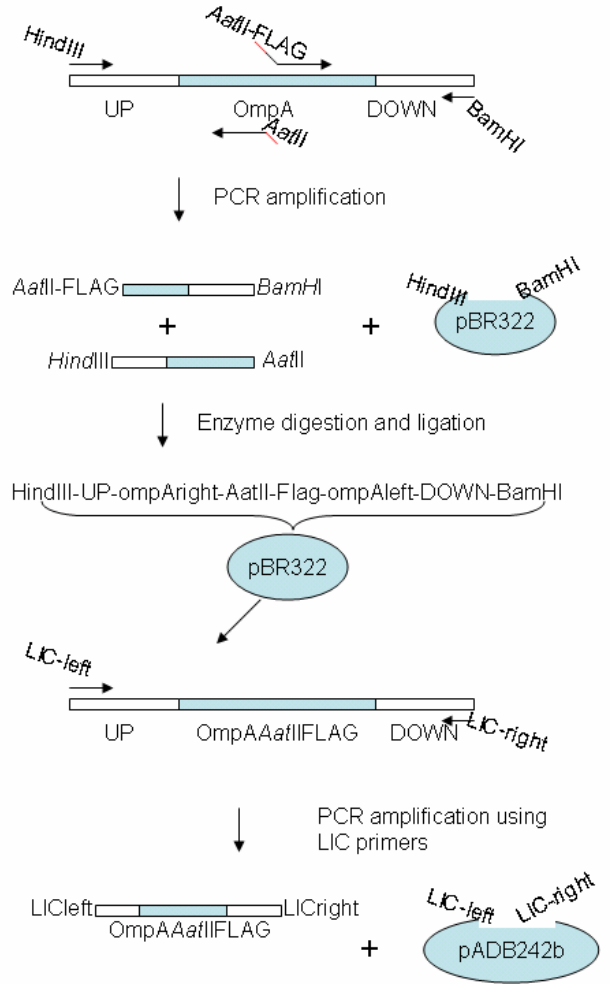


Figure 12: Construction of *ompA1* fragment with Aatii-FLAG sites inserted

Task 3c. Evaluate OmpA1 in a cytokine induction model. The parental OmpA1 has been evaluated in a cytokine induction model and the results are described in the attached publication [14]. Very briefly, the ability of the B. fragilis OmpA1 to trigger release and expression of proinflammatory and immunoregulatory cytokines IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 by murine splenocytes in vitro was measured. Both native and refolded recombinant *B. fragilis* Bf-OmpA1 that are proved to be functionally identical [36] could elicit the cytokine release. The main reason for using recombinant refolded Bf-OmpA1 was to avoid the possibility that any capsule contamination in the purified protein preparation may be responsible for the cytokine production [35]. The cytokine release proceeded in a dose-dependent fashion with different concentrations of porins needed for the maximum release of each cytokine.

Task 3d: Express modified *ompA1* genes generated in Tasks 3a and 3b in *E. coli* and in *B. fragilis*.

Our original assumption was that it would be easier to do all of the modifications in *E. coli*, and then transfer the modified *ompA1* gene to a shuttle to be expressed in *B. fragilis*. Since the heterologous OmpA1 was toxic to *E. coli*, we decided that it would be more efficient and that the results would be more meaningful doing the allelic exchange experiments (described below in Task 4b) with the modified OmpAs.

♦Task 4: Construct *B. fragilis* strains with modified OmpAs by allelic exchange using a double crossover technique.

Task 4a. Construct plasmid to be used in allele exchange. √ Completed

INTRODUCTION TO AIM 4: This aim, along with Aims 1a and 1b constitute the major thrust of this project. The molecular biology tools, kits, databases and services available for work in aerobic organisms is a universe apart from the reality of genetic work with *Bacteroides*. There are only a handful of laboratories doing any kind of molecular biology with *B. fragilis*, and the vectors available are very limited. We are in collegial relationships with the other groups (Dr. C.J. Smith, East Carolina University, and Dr. Michael Malamy, Tufts University); they have provided these vectors to us and these were the basis of our work. There are a few rate limiting steps in our proposed project related to the particular suicide vector that needs to be used for gene insertion, deletion or replacement in *B. fragilis* (pADB242 or derivatives). The plasmid is quite large and the standard kits available for modifying cloned genes for the smaller *E. coli* vectors (e.g. the QuikChange Site-Directed Mutagenesis kit described in the original proposal) did not work with the suicide vector that we were using. Also, the cloning efficiency with such a large vector; this, coupled with the fact that the standard ligase reaction also severely reduced the efficiency of our cloning, resulted in very low recovery of the desired clones. Therefore, we set about modifying the vectors needed so that we could easily obtained the necessary clones.

Construction of the pADB242c vector for use in ligation independent cloning (LIC) protocols. We constructed modified *ompA*s (with the added AatII restriction site, and with the AatII-FLAG epitope and with the AatII-6His tag added before loop 2) in the pADB242c destination vector. We have reinserted the modified *ompA1* gene as outlined in Figure 4. The

construction of the modified suicide vector used in the Ligation-Independent Cloning of the tagged ompAs is described in the next section.

M**odification of pADB242c suicide vector for use in ligation independent cloning. .**

Ligation independent cloning: We have modified the pADB242c suicide vector to expedite construction of the vector+insert for the two step recombination used for the construction of our deletants. As mentioned, we found that limiting step in our construction of deletants was the initial construction of the pADB242c vector containing the upstream and downstream sequences. The large size of the pADB242c results in lower transformation efficiencies than those of smaller vectors typically used. Also, we found that the ligase mediated cloning procedures also significantly decreases the transformation efficiency. Therefore, we have constructed a modified pADB242c vector that incorporates a ligase independent cloning (LIC site) [5]. Because we also found that the background transformation resulting in colonies with plasmid without insert hampered our work, we added the ampicillin resistance gene in the cloning procedure so that we would retrieve only the plasmids that had incorporated the LIC site. Briefly, we amplified the ampicillin resistance gene from puc19, using primers with tails that included respectively, HindIII-LIC sequence-Not1 and Not1-LICsequence-BamH1. The amplicon was added to HindIII /BamH1 digested pADB242c, transformed in *E. coli* Top10 and selected on ampicillin plus chloramphenicol. The resultant plasmid was digested with Not1 and then T4 polymerase with only dTTP added, resulting in the long tails used in LIC; the amplicon containing the LIC tails and the insert gene was similarly digested with T4 polymerase, and dATP, resulting in the complementing LIC tails (Figure 5.) Results indicate that this vector significantly increases the efficiency of obtaining our desired constructs.

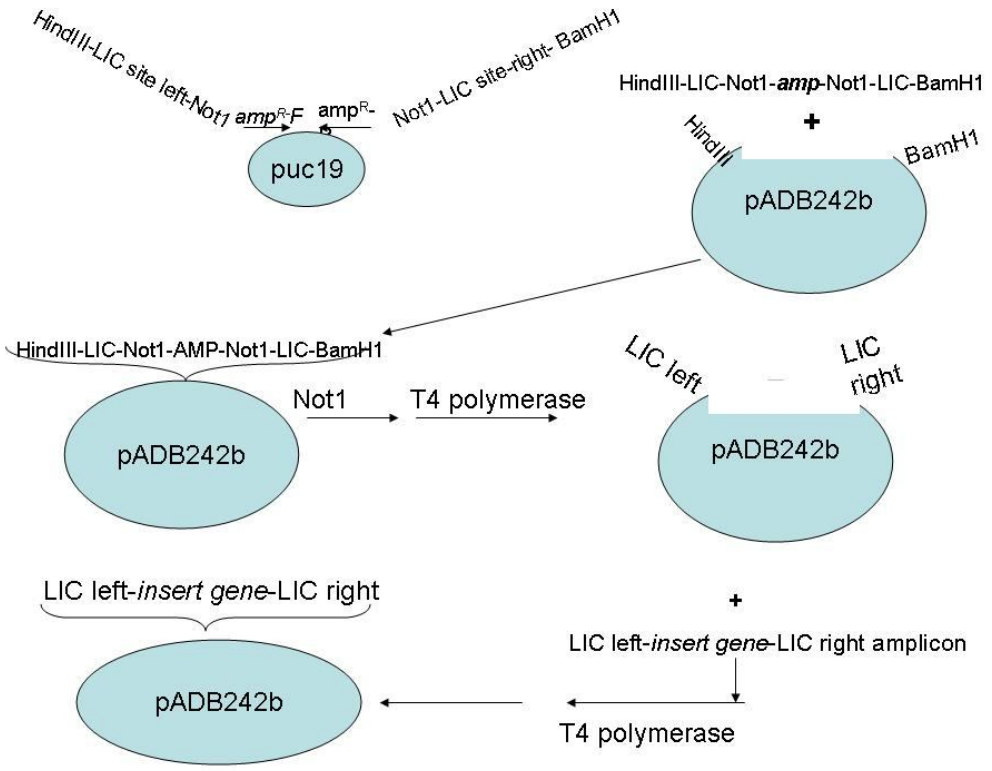


Figure 13. Construction of the modified pADB242b (and pADB242c) suicide vectors for use in Ligation-Independent Cloning (LIC).

Insertion of the modified OmpA1 into the LIC-pADB242b by a ligation independent cloning technique. The modified OmpA1 constructed as described in Task 3b was inserted into the LIC-adapted pADB242c as depicted below. The plasmid was digested with Not1 and then T4 polymerase with only dTTP added, resulting in the long tails used in LIC; the amplicon containing the LIC tails and the insert gene was similarly digested with T4 polymerase, and dATP, resulting in the complementing LIC tails (below). Results indicate that this vector significantly increases the efficiency of obtaining our desired constructs.

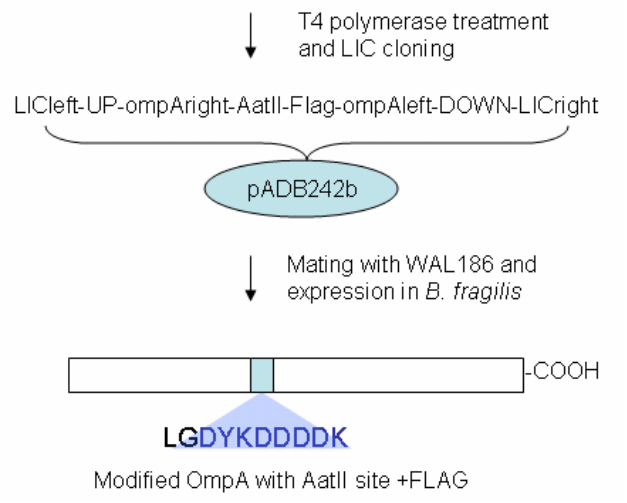
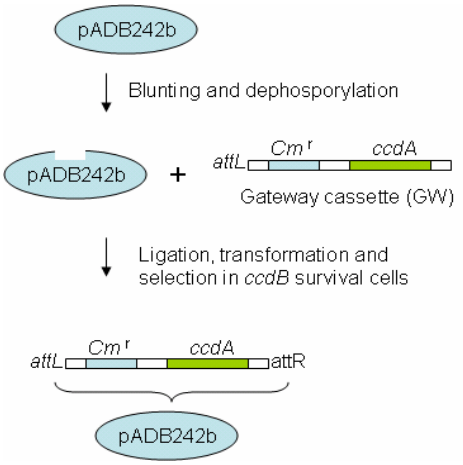


Figure 14: Insertion of the modified OmpA1 into the LIC-pADB242b by a ligation independent cloning technique

Construction of a Gateway-pADB242b suicide vector for use in gene insertions or swapping. As another approach to cloning a modified ompA1 gene, we constructed a Gateway Destination Vector based on the pADB242b suicide vector (Figure 6) using the Invitrogen Gateway technology. Briefly, we ligated the Gateway (GW) Cassette into the digested, blunted and dephosphorylated pADB242b, used the ligation mixture to transform *ccdB* competent cells, and screened the colonies that grew on LB with chloramphenicol. We also cloned the full-length *ompA1* gene into the pCR-TOPO- Entry Vector. Incubation of the entry and destination vectors in the presence of Clonase Enzyme mix will facilitate the recombination at the corresponding attLR sites present in both vectors, resulting in the efficient transfer of the gene insert to the destination vector.

Figure 15. Construction of the modified pADB242b (and c) suicide vectors for use in Gateway Cloning



Task 4b. Construct *B. fragilis* with modified OmpA. √ Completed

We modified our original plan for constructing these strains and used *WAL186 ΔompA1* (constructed during the first phase of these studies) as the parental strain. First, using the same two step recombination procedure that was used to construct that strain, we replaced the missing gene with a cloned *ompA1* to confirm that the newly constructed strain still expresses *ompA1* properly. This technique has several advantages: The only strains with an OmpA1 protein will have the modified OmpA1 protein, since we are beginning with a strain lacking this protein.

Construction of WAL 360 + *ompA1* (i.e., *ompA1* reinsertant). The full-length *B. fragilis ompA1* gene (including about 800 base pairs upstream and downstream of the gene) was cloned in the suicide vector pADB242b. The recombinant plasmid was verified by DNA sequencing. *E.coli DH5α*/pADB242b-up*ompA1*down and *E.coli DH5α /pRK231* were mated with *B. fragilis* WAL 186 Δ*ompA1* as described [1] and the cointegrants selected as described above. Cointegrants were plated on minimal media with thymine and trimethoprim to select for the second recombination event. Reinsertants containing full length *ompA1* were confirmed by sequencing and the *ompA1* "reinsertant" was named WAL 360 + *ompA1*.

PCR analysis and sequencing of the putative insertion site confirmed proper insertion by DNA sequencing. SDS-PAGE analysis of the outer membrane confirmed that there are no differences between the parent strain and the deletant strain in which the *ompA1* gene was reinserted and that the OmpA1 protein is indeed translated.

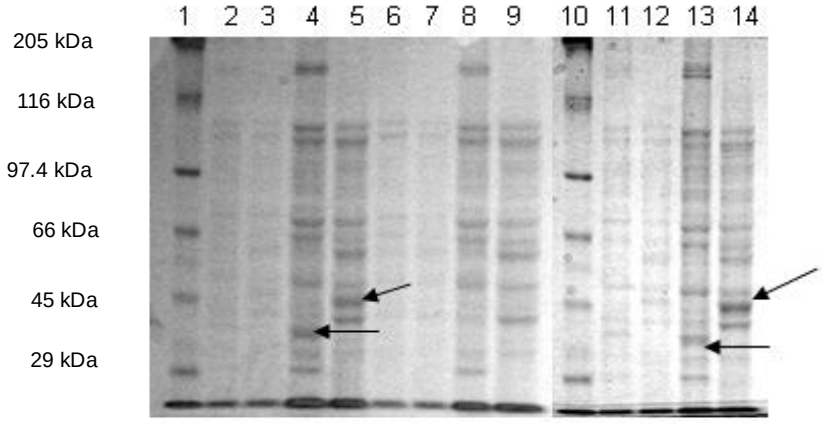


Figure 16: **SDS-PAGE analysis of OmpA parental, deletant and reinsertant strains.** Figure 3 is a composite of two gels run simultaneously. Lane 1 and Lane 10: Molecular weight (MW) markers 205, 116, 94.7, 66, 4 and 29 kDa, respectively; 2 and 3: *B. fragilis* WAL 108 wild type cell lysates; 4 and 5: WAL 108 wild type Triton pellet (25'C and boiled, respectively); 6 and 7: WAL 186 Δ*ompA1* cell lysates; 8 and 9: WAL 186 Δ *ompA1* Triton pellet (25'C and boiled, respectively); 10: MWmarkers; 11 and 12: WAL 360 + *ompA1* cell lysates; and 13 and 14: WAL 360 +*ompA*1 Triton pellet (25'C and boiled, respectively).

Construction of B. fragilis reinsertant with FLAG-tagged OmpA. DH5alpha containing the pADB242c::*ompA*-AatIIFLAG (and including about 800 base pairs upstream and downstream of the gene) was used in a three part mating with *E.coli DH5α* /*pRK231* and *B. fragilis* WAL 186 Δ*ompA1* as described above. Cointegrants selected as described above, and then plated on minimal media with thymine and trimethoprim to select for the second recombination event. Reinsertants containing *ompA1*-AatIIFLAG were confirmed by restriction analysis *B. fragilis* with the *ompA-AatIIFLAG* gene "reinserted" was named WAL 410+*ompA1*/AatII-FLAG.

Task 4c. Confirm insertion of FLAG tag in *B. fragilis* WAL 410+*ompA1*/AatII-FLAG. Insertion of the FLAG tag in the *ompA1* gene was confirmed by restriction digest analysis as described above. As this report is submitted, we are finishing the very last experiment in this protocol. As mentioned, we have already constructed the *B. fragilis* strain with the OmpA1 protein modified with FLAG. We are using FLAG antibody detection method to demonstrate that the tag is properly situated and exposed on the outside loop. Unfortunately, we discovered that while the kits we purchased for this use was within the usable dates, several of the components in the kits were almost 10 years old and expired. We have already ordered new supplies and plan to do this final experiment shortly. Since it will be done quickly, there was no need to ask for an extension, but this experiment will be included in the publication that will emanate from this work, so we wanted to ensure that it is mentioned in the final report.

Task 4d. Compare *B. fragilis* with loop-deleted OmpAs to the parental OmpA1 in a mouse abscess model.

Task 4e: Compare parental and *ompA1* deletant strains in an invasion assay of brain microvascular endothelial cells.

Since there was no difference between the parental strain and the *ompA1* deletant in the mouse abscess model, this task does not need to be completed. Similarly, since both the OmpA1 deletant (WAL 186) and the parental strain (WAL 108) were deficient in 1) adherence to intestinal epithelial cells and 2) biofilm formation compared to strain 638R (WAL 108 is a *thy* $^-$ deficient mutant of 638R, engineered to be more amenable to use in allelic exchange experiments) (Task 1D), there was no further need to test specific loop deletants.

KEY RESEARCH ACCOMPLISHMENTS

♦Construction of *ompA1* deletant strain

♦Characterization of *ompA1* deletant strain WAL186ΔompA1

♦Transcriptome analysis of WAL186Δ*ompA*1

♦♦ **Construction of vectors optimized for rapid construction of epitope specific *ompA* genes**

Modification of pADB242b vector to remove AatII restriction site

LIC (ligation independent cloning) pADB242b and pADB242c vectors

Gateway adapted pADB242b and pADB242c vectors

♦Modification of *B. fragilis ompA1*

Insertion of restriction specific sites

Insertion of AatII-FLAG

Insertion of His-TAG

♦Construction of WAL360 ompA1 reinsertant strain and confirmation of proper expression of OmpA1

♦Construction of WAL410 FLAG-tag *ompA1* reinsertant strain

REPORTABLE OUTCOMES

Wexler, Hannah M. , *Bacteroides fragilis* Omp- Utility as a live vaccine vector for biodefense agents: Construction of an ompA deletant and characterization of the function of OMPA. Presented to PRMRP Military Health Research Forum, Puerto Rico, May 2006.

Magalashvili, L., S. Lazarovich, I. Pechatnikov et al. 2008. Cytokine release and expression induced by OmpA proteins from the Gram-negative anaerobes, Porphyromonas asaccharolytica and Bacteroides fragilis. FEMS Immunol.Med.Microbiol. **53**:252-259.

Wexler, H. M., L. Pumbwe, and E. Tenorio. 2009. Characteristics of *Bacteroides fragilis* lacking the major outer membrane protein, OmpA. Microbiology, revisions requested and resubmitted.

Tenorio, E., Chan, K., Minato, Y., Patrick, S. and Wexler, H.M. 2009. Deletion of the major outer membrane protein *ompA* gene causes a generalized stress response in *Bacteroides fragilis.* Manuscript in preparation.

Submission of transcriptome data to GEO (Gene Expression Omnibus) at the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH), Washington, D.C.

CONCLUSION

This project was initiated to explore the use of the *B. fragilis* major outer membrane protein, OmpA, for use as a carrier for specific epitopes, including those that might be used in bioterror attacks. The modified *ompA* gene would then be inserted into a modified *B. fragilis* lacking this protein, and the modified *B. fragilis* would then be used as a live vaccine. As a human gut commensal, the modified *B. fragilis* strain could colonize the mucosal tissues in the gut and elicit an immune response.

In order to accomplish these aims, we had to optimize the tools available for inserting genes into the *B. fragilis* chromosome. Unlike the massive numbers of kits and other services available for *E. coli*, there are only a few vectors available for genetic manipulation in *B. fragilis* and the efficiency of obtaining the desired clone was not adequate for our needs. We therefore redesigned these vectors, resulting in two improved ways for us to insert the desired gene into *B. fragilis*. These included the suicide vector with a ligation independent cloning site, and a suicide vector with a GATEWAY cassette insert (this allows cloning without digestion or ligation). At this point, we can quickly assemble the desired fragments and insert them into the suicide vector.

REFERENCES

1. **Baughn, A. D. and M. H. Malamy**. 2002. A mitochondrial-like aconitase in the bacterium *Bacteroides fragilis*: implications for the evolution of the mitochondrial Krebs cycle.PG -. Proc.Natl.Acad.Sci.U.S.A **99**.
2. **Bayley, D. P., E. R. Rocha, and C. J. Smith**. 2000. Analysis of *cepA* and other *Bacteroides fragilis* genes reveals a unique promoter structure. FEMS Microbiol.Lett. **193**:149-154.
3. **Benjamini, Y. and Y. Hochberg**. 2005. *Controlling the false discovery rate: a practical and powerful approach to multiple testing*. Journal of the Royal Statistical Society, Series B (Methodological) **57**:125-133.
4. **Bolstad, B. M., R. A. Irizarry, M. Astrand et al.** 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. **19**:185-193.
5. **Cabrita, L. D., W. Dai, and S. P. Bottomley**. 2006. A family of E. coli expression vectors for laboratory scale and high throughput soluble protein production. BMC.Biotechnol. **6**:12.
6. **Cerdeno-Tarraga, A. M., S. Patrick, L. C. Crossman et al.** 2005. Extensive DNA inversions in the *B. fragilis* genome control variable gene expression. Science **307**:1463-1465.
7. **Chen, R., W. Schmidmayr, C. Kramer et al.** 1980. Primary structure of major outer membrane protein II (ompA protein) of *Escherichia coli* K-12. Proc.Natl.Acad.Sci.U.S.A **77**:4592-4596.
8. **Glaser, F., T. Pupko, I. Paz et al.** 2003. ConSurf: identification of functional regions in proteins by surface-mapping of phylogenetic information. Bioinformatics. **19**:163-164.
9. **Imai, M., Y. Murakami, K. Nagano et al.** 2005. Major outer membrane proteins from Porphyromonas gingivalis: strain variation, distribution, and clinical significance in periradicular lesions. Eur.J.Oral Sci. **113**:391-399.
10. **Irizarry, R. A., B. Hobbs, F. Collin et al.** 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. **4**:249-264.
11. **Iwami, J., Y. Murakami, K. Nagano et al.** 2007. Further evidence that major outer membrane proteins homologous to OmpA in Porphyromonas gingivalis stabilize bacterial cells. Oral Microbiol.Immunol. **22**:356-360.
12. **Kuwahara, T., A. Yamashita, H. Hirakawa et al.** 2004. Genomic analysis of *Bacteroides fragilis* reveals extensive DNA inversions regulating cell surface adaptation. Proc.Natl.Acad.Sci.U.S.A **101**:14919-14924.
13. **Landau, M., I. Mayrose, Y. Rosenberg et al.** 2005. ConSurf 2005: the projection of evolutionary conservation scores of residues on protein structures. Nucleic Acids Res. **33**:W299-W302.
14. **Magalashvili, L., S. Lazarovich, I. Pechatnikov et al.** 2008. Cytokine release and expression induced by OmpA proteins from the Gram-negative anaerobes, Porphyromonas asaccharolytica and Bacteroides fragilis. FEMS Immunol.Med.Microbiol. **53**:252-259.
15. **Magalashvili, L., I. Pechatnikov, H. M. Wexler et al.** 2007. Isolation and characterization of the Omp-PA porin from *Porphyromonas asaccharolytica*, determination of the omp-PA gene sequence and prediction of Omp-PA protein structure. Anaerobe.
16. **Murakami, Y., M. Imai, H. Nakamura et al.** 2002. Separation of the outer membrane and identification of major outer membrane proteins from *Porphyromonas gingivalis*. Eur.J.Oral Sci. **110**:157-162.
17. **Nagano, K., E. K. Read, Y. Murakami et al.** 2005. Trimeric structure of major outer membrane proteins homologous to OmpA in Porphyromonas gingivalis. J.Bacteriol. **187**:902-911.

18. **Onderdonk, A. B., D. L. Kasper, B. J. Mansheim et al.** 1979. Experimental animal models for anaerobic infections. Rev.Infect.Dis. **1**:291-301.

19. **Onderdonk, A. B., W. M. Weinstein, N. M. Sullivan et al.** 1974. Experimental intra-abdominal abscesses in rats: quantitative bacteriology of infected animals. Infect.Immun. **10**:1256-1259.

20. **Prasadarao, N. V., C. A. Wass, M. F. Stins et al.** 1999. Outer membrane protein A-promoted actin condensation of brain microvascular endothelial cells is required for *Escherichia coli* invasion. Infect.Immun. **67**:5775-5783.

21. **Prasadarao, N. V., C. A. Wass, J. N. Weiser et al.** 1996. Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. Infect.Immun. **64**:146-153.

22. **Pumbwe, L., D. Glass, and H. M. Wexler**. 2006. Efflux pump overexpression in multiple antibiotic resistant mutants of *Bacteroides fragilis*. Antimicrob.Agents Chemother. **50**:3150-3153.

23. **Pumbwe, L., D. Glass, and H. M. Wexler**. 2006. Efflux Pump Overexpression in Multiple Antibiotic Resistant Mutants of *Bacteroides fragilis* (BF). Abstr.of the 106th Annu.Meeting, ASM, Orlando, FL.

24. **Pumbwe, L., C. Skilbeck, A. Oren et al.** 2007. Bile salts enhance bacterial co-aggregation, bacterial-intestinal epithelial cell adhesion, biofilm formation and antimicrobial resistance of *Bacteroides fragilis* . Microbial Pathogenesis **43**.

25. **Pumbwe, L., O. Ueda, F. Yoshimura et al.** 2006. *Bacteroides fragilis* BmeABC Efflux Systems Additively Confer Intrinsic Antimicrobial Resistance. J.Antimicrob.Chemother. **58**:37-46.

26. **Rocha, E. R., A. O. Tzianabos, and C. J. Smith**. 2007. Thioredoxin reductase is essential for thiol/disulfide redox control and oxidative stress survival of the anaerobe Bacteroides fragilis. J.Bacteriol. **189**:8015-8023.

27. **Schirmer, T. and S. W. Cowan**. 1993. Prediction of membrane-spanning beta-strands and its application to maltoporin. Protein Science **2**:1361-1363.

28. **Shata, M. T., L. Stevceva, S. Agwale et al.** 2000. Recent advances with recombinant bacterial vaccine vectors. Mol.Med.Today **6**:66-71.

29. **Smith, S. G., V. Mahon, M. A. Lambert et al.** 2007. A molecular Swiss army knife: OmpA structure, function and expression. FEMS Microbiol.Lett. **273**:1-11.

30. **Stintzi, A., D. Marlow, K. Palyada et al.** 2005. Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of Campylobacter jejuni. Infect.Immun. **73**:1797-1810.

31. **Struyve, M., M. Moons, and J. Tommassen**. 1991. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J.Mol.Biol. **218**:141-148.

32. **Sukumaran, S. K., H. Shimada, and N. V. Prasadarao**. 2003. Entry and intracellular replication of Escherichia coli K1 in macrophages require expression of outer membrane protein A. Infect.Immun. **71**:5951-5961.

33. **Vogel, H. and F. Jahnig**. 1986. Models for the structure of outer-membrane proteins of *Escherichia coli* derived from raman spectroscopy and prediction methods. J.Mol.Biol. **190**:191-199.

34. **Wang, Y.** 2002. The function of OmpA in *Escherichia coli*. Biochem.Biophys.Res.Commun. **292**:396-401.

35. **Wexler, H. M.** 2007. *Bacteroides*--The Good, the Bad, and the Nitty-Gritty. Clin.Microbiol.Rev. **20**:593-621.

36. **Wexler, H. M., E. K. Read, and T. J. Tomzynski**. 2002. Identification of an OmpA protein from *Bacteroides fragilis*: *ompA* gene sequence, OmpA amino acid sequence and predictions of protein structure. Anaerobe **8**:180-191.

37. **Woodruff, W. A. and R. E. Hancock**. 1989. *Pseudomonas aeruginosa* outer membrane protein F: structural role and relationship to the *Escherichia coli* OmpA protein. J.Bacteriol. **171**:3304-3309.

38. **Yoshimura, F., Y. Murakami, K. Nishikawa et al.** 2008. Surface components of Porphyromonas gingivalis. J.Periodontal Res.

APPENDICES

Table 1: Strains, plasmids and primers used in this study

Strain or plasmid	Description or relevant marker	Source or reference
B. fragilis		
WAL 3501	NCTC 9343 (aka ATCC 25285)	ATCC
WAL 26	*B. fragilis* 638R	
WAL 108	*B. fragilis* ADB77; An isogen of 638R optimised for genetic manipulation , parental strain of deletion mutant. TM400 thyA-, rif^{R}, tmp$^{R.}$	Baughn & Malamy; [4,5]
WAL 67Ω *ompA1*	*B. fragilis* 638R/pFD516::'*ompA1*''; erythromycin^{R}	This study
WAL 174	*B. fragilis* ADB77/pYT102::*ompA1* updown; tetracycline^{R}	This study
WAL 186 Δ*ompA1*	*ompA* deletant	This study
WAL 360 + *ompA1*	WAL 186 Δ*ompA1* with full length *ompA* reinserted	This study
WAL 410 + *ompA1*	WAL 186 Δ*ompA1* with full length *ompA* + *Aat*II-FLAG reinserted	This study
E. coli		
DH5α	Δ *lacZ* M15	Baughn & Malamy; [4,5]
S17-1	DW1030; host strain for pRK231	Baughn & Malamy; [4,5]
WAL 34	*E. coli* DH5α/pFD516::'*ompa*'; erythromycin^{R}	this study
WAL 169	*E. coli* DH5α/pYT102::*ompA1*updown; tetracycline^{R}	this study
WAL 310	*E coli/pAD242b::up-ompA1-down*	this study
WAL 398	*E coli/pAD242b:: up-ompA1-AatII-down*	this study
WAL 399	*E coli/pAD242b:: up-ompA1-AatII-FLAG-down*	this study
WAL 402	*E coli/pAD242b::LIC- up-ompA1-AatII-FLAG-down*	this study
WAL 406	*E coli/pAD242b::LIC- up-ompA1-AatII-6His-down*	this study
Plasmids		Baughn & Malamy; [4,5]
pFD516		Smith ***
pFD516::'*ompA1*'	pFD516 with 361 bp of internal *ompA* sequence	
pTY102	P15 *ori*, chloramphenicol^{R}, RP4 *oriT*; *B. fragilis* suicide vector, *thyA*$^{+}$, tetracycline^{R}	Baughn & Malamy; [4,5]
pTY102::'*ompA1*updown"	pYT102 with 800bp upstream and 800 bp of downstream ompA sequence	
pADB242b	pYT102 derivative, thy defective, tet^{R}	Baughn & Malamy; [4,5]
pAD242b::up-*ompA1*-down	pADB242b with full length *ompA* and 800 bp of upstream and	

	downstream sequence	
pRK231	RP4 derivative, tetracycline^{R}, ampicillins; broad host –range mobiliser plasmid	Baughn & Malamy; [4,5]
pADB242b::GWdest	pADB242b with Gateway Cassette (for use as a destination vector in Gateway Cloning)	this study
pADB242b::LICamp	pADB242b with LICleft-*bla*-LICright (for use in Ligation-Independent Cloning	this study

Primers
Restriction endonuclease sites are underlined.

Primer name	**Primer sequence 5' to 3'**	**Reference**
Plasmid specific primers		
pRY102-forward (61 RAB)	ggcgcgccgtaaggaaagtggctctcag	Baughn & Malamy; [4,5]
pRY102-reverse (1843)	ccatcggtgatgtcggc	Baughn & Malamy; [4,5]
Primers for constructing ompA variant strains		this study
pFD516::'ompA1-internal'F	cagctgattttgtgaagagtgg	this study
pFD516::'ompA1internal'R	cccgggaaacgataagtcaaa	this study
pTY102::'ompA1updown"-UpF	aaggaagcttacagtctgggagtcgctacct	this study
pTY102::'ompA1updown"-UpR	cgttccatggtcccgtaatcgtagtctgctg	this study
pTY102::'ompA1updown"-DownF	ccagccatggtggtggtgtggataaattcg	this study
pTY102::'ompA1updown"-DownR	gcttggatcctcacttaccccatcagtcagg	this study
pAD242b::up-ompA1-down-F	aaggaagcttacagtctgggagtcgctacct	this study
pAD242b::up-ompA1-down-R	gcttggatcctcacttaccccatcagtcagg	this study
Primers for RT PCR		
OmpA1-F	gga tat gac ggt gtt gcc ag	this study
OmpA1-R	tag cag cag cca tgt cat tc	this study
OmpA2-F	tag aag gtg cat gga cta ct	this study
OmpA2-R	aac cgc caa tag cat tgg ac	this study
OmpA3-F	act ccg ctg atc aat gtg tc	this study
OmpA3-R	cgt ctg cac gca tag tga ag	this study
OmpA4-F	cca aga tcg acg act atg ct	this study
OmpA4-R	ttc tgg ttc cac ttg gca ct	this study
Primers for qRT PCR analysis of ompAs1-4		
OmpA1-F-q	cagctgattttgtgaagagtgg	this study
OmpA1-R-q	cccgggaaacgataagtcaaa	this study
OmpA2-F-q	caaccccgacaactttgatt	this study
OmpA2-R-q	taggccttggcaaacgtaag	this study
OmpA3-F-q	tttcatgccgacactttctg	this study
OmpA3-R-q	acggtaacgtgcctggatac	this study
OmpA4-F-q	ggacagcctgccgatactta	this study
OmpA4-R-q	ttaccaaacggagacggaga	this study

Primers for constructing modified pADB242b suicide vectors		
HindIIILICNotApF	cgagctaagcttcgccagggagcagcggccgccgaaagggcctcgtgatac	this study
BamHILICNotApR	cgagctggatccgcaaaggagcggccgccagcagattacgcgcagaa	this study
Primers for constructing modified ompA1s		
Loop2AatIIFor	gcttatgacgtcggctttgccggttcggaa	this study
Loop2AatIIRev	gctgatgacgtcacgtccctgtaagccactg	this study
Loop2AatIIFlagF	gcttatgacgtcgattacaaagatgatgatgataaaggctttgccggttcggaa	this study
Loop2AatIIHisF	gcttatgacgtccatcatcaccatcaccacggctttgccggttcggaa	this study
Loop3SalIFor	gcatgaggtcgacgaggcgtttgctatgaac	this study
Loop3SalIRev	gctcctggtcgacacgatgaggtttcgaata	this study
229LICompAF	ccgcaaaggagcggcctacagtctgggagtcgctacct	this study
222LICompAR	ccgcaaaccagcggccttcacttaccccaatcagtcagg	this study

RESEARCH ARTICLE

Cytokine release and expression induced by OmpA proteins from the Gram-negative anaerobes, *Porphyromonas asaccharolytica* and *Bacteroides fragilis*

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Porphyromonas asaccharolytica; *Bacteroides fragilis*; OmpA; cytokine.

Abstract

OmpA proteins from Gram-negative anaerobes *Porphyromonas asaccharolytica* and *Bacteroides fragilis* induced release and expression of IL-1α, tumor necrosis factor (TNF)-α, IFN-γ, IL-6, and IL-10 from murine splenocytes *in vitro* in a dose-dependent fashion. The release of the cytokines induced by *B. fragilis* Bf-OmpA was at much lower levels compared with *P. asaccharolytica* Omp-PA; Bf-OmpA did not induce release of IL-10. Omp-PA and Bf-OmpA were able to upregulate mRNA expression of the tested cytokines. The results obtained with refolded Bf-OmpA were similar to those with native Bf-OmpA. The data presented in this research demonstrate for the first time that Omps from anaerobic bacteria can induce the release of cytokines, suggesting that Omp-PA and Bf-OmpA may play important roles in the pathogenic processes of these bacteria.

Introduction

Porphyromonas asaccharolytica is a Gram-negative nonsporulating anaerobic rod, that was formerly part of the genus *Bacteroides* (Nitzan *et al*., 1999). Infections by this pathogen are associated with soft-tissue infections below the waist, foot ulcers, appendiceal abscesses, and empyema (Jousimies-Somer *et al*., 2002). *Porphyromonas asaccharolytica* was also implicated in cases of bacteremia (Melon *et al*., 1997) and in a left cardiac myxoma (Revankar & Clark, 1998). *Bacteroides fragilis*, a nonspore-forming, Gram-negative rod, is the most common anaerobic organism isolated from clinical infections. It is frequently associated with extraintestinal infections such as abscesses and soft-tissue infections, as well as diarrheal diseases in animals and humans. *Bacteroides fragilis* has the ability to invade the host immune response, which contributes to the virulence of the bacterium. Its capsule can mediate resistance to complement-mediated killing and to phagocytic uptake (Wexler, 2007).

The outer membrane of Gram-negative bacteria acts as a dynamic interface between the cell and its surroundings, and the importance of this interaction in both pathogenesis of the infection and immune response of the host has been investigated. The major components of the outer-membrane proteins in *P. asaccharolytica* and *B. fragilis* are proteins of the OmpA family, Omp-PA and Bf-OmpA, respectively. The importance of OmpA in the pathogenic process has been increasingly recognized. OmpA has been implicated in the invasion of brain microvascular endothelial cells (BMEC) (Prasadarao *et al*., 1996, 1999) and has been shown to contribute to the ability of *Escherichia coli* to cross the blood–brain barrier (Huang *et al*., 2000).

The outer-membrane proteins of the porin class, in general, possess a variety of immunomodulatory and procoagulant activities (Gupta, 1998; Iovane *et al*., 1998; Biswas, 2000; Brinkman *et al*., 2000). Omp-PA of *P. asaccharolytica* is the major porin protein of that organism (Magalashvili *et al*., 2007). In contrast, the Bf-OmpA protein does possess some pore-forming activity in liposomes but is not the

major porin protein of *B. fragilis* (Wexler *et al.*, 2002). Nontoxic concentrations of porins from a variety of organisms stimulate the synthesis and release of platelet-activating factor and promote proinflammatory and immunomodulatory cytokine release from immunocompetent cells or other cellular sources (Perfetto *et al.*, 2003). Porins can induce the release of tumor necrosis factor (TNF)-α, IL-1α, and IL-6 by human monocytes and of IFN-γ and IL-4 by human lymphocytes. This was seen with porins from *Salmonella typhimurium* (Galdiero *et al.*, 1993, 1995; Gupta, 1998), *Pseudomonas aeruginosa* (Brinkman *et al.*, 2000; Perfetto *et al.*, 2003), and *Pasteurella multocida* (Iovane *et al.*, 1998). The OmpA-like porin from *Acinetobacter* spp. stimulates the secretion of gastrin and IL-8 (Ofori-Darko *et al.*, 2000), and *Shigella dysenteriae* type 1 porin induces the release of nitric oxide and IL-1 (Biswas, 2000). The accumulated evidence clearly indicates that porins mediate release of cytokines and other proinflammatory factors, and that this activity may vary from one porin type to another.

The aim of this study was to investigate and compare the abilities of the OmpA proteins *P. asaccharolitica* and *B. fragilis* to induce the release of different cytokines by murine splenocytes *in vitro*. The significance of this study is due to the fact that *Bacteroides* and anaerobes in general are quite different from other aerobic microorganisms.

Materials and methods

Animals

BALB/cByJ male mice, 8 weeks old were used. The animals were housed at a constant temperature (20 ± 2 °C) under a fixed 12 h light–dark cycle with free access to food and water.

Preparation of Omp-PA and Bf-OmpA

Porphyromonas asaccharolytica ATCC 25260 and *B. fragilis* ATCC 25285 were grown anaerobically for 72 and 96 h, respectively, at 37 °C in Brucella broth medium supplemented with hemin and vitamin K (5 and 1 μg mL^{-1}). *Porphyromonas asaccharolytica* Omp-PA and *B. fragilis* OmpA isolation and purification from the outer membranes were performed using lithium dodecyl sulfate (LDS) as described earlier (Nitzan *et al.*, 1999; Wexler *et al.*, 2002; Magalashvili *et al.*, 2007). Lipopolysaccharide contamination in the final preparations was detected by the Limulus test (Yin *et al.*, 1972) and was 10 pg per 10 μg porins. To eliminate any biological effect of lipopolysaccharide, proteins were incubated for 1 h with 5 μg mL^{-1} polymyxin B (Sigma) at room temperature (Blanchard *et al.*, 1986). In all of the tests performed, the porin with polymyxin B gave the same results as the porin alone (data not shown).

The supernatants containing the OmpA proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970).

Elution from the gel

In order to obtain purified preparations containing only Omp-PA (37 kDa) and Bf-OmpA (37 kDa) in their native forms, elution from the gel was performed as follows:. bands corresponding to the proteins were cut out from the gel, placed in dialysis bags, and mashed manually. Buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, and 3 mM sodium azide was then added and the preparations were dialyzed against the same buffer overnight at 4 °C. The gel/buffer mixtures were centrifuged to remove the gel traces, which were re-extracted with the same buffer and recentrifuged. In order to remove the detergent traces, the supernatants from both extractions were combined and redialyzed against buffer containing 20 mM Tris–HCL and 3 mM sodium azide for 4 days at room temperature, with frequent changes of the buffer, and then lyophilized.

Cloning and expression of *B. fragilis* ompA in *E. coli* and purification of recombinant Bf-OmpA from inclusion bodies

Primers, to amplify the Bf-*ompA* gene from genomic DNA, were constructed with appropriate restriction sites (NdeI and BamH1) for subsequent cloning of the PCR product into pET-27b(+). The primers used are indicated in Table 1. The cycles were 95 °C for 3 min, followed by 30 cycles of 95 °C for 45 s, 62 °C for 30 s, 72 °C for 4 min, and finally 72 °C for 5 min. One microgram each of plasmid and PCR product were digested with BamHI and NdeI as directed by the manufacturer. Both the digested pET-27b(+) and the digested Bf-*ompA* were purified from a 1% agarose gel. The gel slices containing the desired bands were melted at 65 °C for 5 min. Fifty nanograms of digested vector and 100 ng of digested insert were mixed with 400 U of T4 ligase in T4 ligase buffer (20 μL total volume) for 4.5 h. Five microliters of the ligation mixture was used to transform XLBlue MRF' and the reaction was plated on Luria–Bertani (LB) agar with kanamycin. Transformants were screened by PCR and verified by miniprep plasmid analysis.

Purified plasmid DNA was used to transform BL21 according to the manufacturer's instructions and plated on LB agar with kanamycin. Overnight culture (0.5 mL) was used to inoculate 1 L of LB with kanamycin and grown in a 37 °C shaker until the $OD_{600\,nm}$ reached 0.4 U. The culture

Table 1. Primers used for the pET cloning

	Primer sequence (5′–3′)
OmpAfwd	TGTTCATATGCAGCAGACTACAATTACGGGAT
OmpArev	AAGTGGATCCTTATTTAACAGACTCTACTAATA

was induced at this point by adding IPTG to a final concentration of 1 mM. Protein induction proceeded for 3 h, and then the cells were harvested by centrifugation for 20 min at 7450 ***g*** at 4 °C. The cell pellet was washed with 20 mM Tris-HCl, pH 7.4, recentrifuged, and then frozen and stored at − 20 °C.

The frozen cell pellet from 1 L of induced culture was resuspended in 100 mL of 20 mM Tris-HCl, pH 7.4, with 100 μg mL^{-1} lysozyme. The suspension was sonicated at a power level of 4 for 9 min until homogenized. The lysate was centrifuged for 27 000 ***g*** for 5 min at 4 °C to pellet the inclusion bodies containing the recombinant Bf-OmpA. The inclusion bodies were washed twice with 100 mL of 20 mM Tris-HCl, pH 7.4, supplemented with 10 mM EDTA, 1%Triton X-100. The washed inclusion bodies were frozen at − 20 °C.

Refolding of overexpressed Bf-OmpA

The frozen inclusion bodies were resuspended to 20 mg mL^{-1} (0.48 mM) in 8 M urea, 10 mM borate, 2 mM EDTA, pH 10. Twelve milligrams of the resuspended protein was incubated with 16 mM 3–14 zwittergent for 4 days at 37 °C. A band indicating the refolded Bf-OmpA was cut out of the gel, passively eluted, and then diluted in the cell culture at the appropriate concentration (0.1 ± 10 mg mL^{-1}) for the cytokine assays.

Murine splenocyte preparation and stimulation

Murine splenocytes were prepared according to conventional procedures from aseptically removed mouse spleens (known to contain more than 97% lymphocytes of different types). Erythrocytes were lysed using 0.155 M NH_4Cl, washed three times in RPMI 1640 medium (Labtek Laboratories, Eurobio, Paris, France), resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), glutamine (2 mM), penicillin (100 U mL^{-1}), and streptomycin (100 U mL^{-1}) at a concentration of 3×10^6 cells mL^{-1}, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Murine splenocytes resuspended at 3×10^6 cells mL^{-1} in complete medium were divided into aliquots to be treated or left untreated. The proteins were prepared in pyrogen-free distilled water and then diluted in the cell culture at the appropriate concentration (0.1 ± 10 μg mL^{-1}). The incubation time was 24 and 48 h for cytokine assays and 3 h for mRNA analysis.

After incubation times were completed a lactate dehydrogenase (LDH) assay was carried out according to the manufacturer's instructions using a Cytotoxicity Detection kit (Promega). LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into cell culture supernatant upon damage of the plasma membrane. LDH activity was determined by a coupled enzymatic reaction whereby the tetrazolium salt (INT) was reduced to formazan. An increase in the number of dead or damaged cells results in an increase in LDH activity in the culture supernatant.

Cytokine assays

All assays were carried out with 3×10^6 cells mL^{-1} stimulated with various concentrations of Omp-PA, native Bf-OmpA, and recombinant Bf-OmpA, and were incubated at 37 °C in 5% CO_2 for 24 and 48 h. At specified time intervals, cell viability was checked by the Trypan blue exclusion test. Culture supernatants were harvested by centrifugation and stored at − 20 °C until assayed for cytokines. All measurements were carried out using monoclonal antibodies. IL-1α, IL-6, TNF-α, IFN-γ, and IL-10 were measured using immunoenzymatic methods (ELISA kits of Invitrogen, Biosource, Worcester, MA).

RNA isolation and cDNA preparation

The concentrations of Omp-PA used in the assay were 0.1 μg mL^{-1} for TNF-α, 1 μg mL^{-1} for IFN-γ and IL-10, and 5 μg mL^{-1} for IL-1α and IL-6. The concentrations of native and recombinant Bf-OmpAs used in the assay were 0.1 μg mL^{-1} for IL-1α and IFN-γ, 1 μg mL^{-1} for TNF-α, and 5 μg mL^{-1} for IL-6. Nonstimulated cells were used as negative controls. The stimulated and the nonstimulated cells were collected after 3 h of incubation. Mouse β-actin was used as an internal standard. Total RNA was extracted according to the method of Chomczynski & Sacchi (1987). The RNA pellet was resuspended in 75% ethanol, sedimented, vacuum-dried, and dissolved in 50 μL of RNAse free water. One microliter of oligo (dT) (Promega Biotechnology, Madison, WI) was added to the suspension containing 2 μg of RNA and the mixture was heated at 70 °C for 5 min. After cooling on ice, the mixture was incubated for 2 h at 42 °C with 14 μL of the following mixture: 20 mM dithiothreitol (Sigma, St Louis, MO); 1 mM (each) dATP, dGTP, dCTP, and dTTP; 35 U of RNasin (Promega); and 525 U of Moloney murine leukemia virus reverse transcriptase (Promega) in reverse transcription buffer.

PCR procedure

The primer pair sequences were designed on the basis of published cytokine gene sequences as reported in Table 2. The primer sequences were complementary to sequences in the exons or spanned exon ± exon junctions and thus were RNA-specific. One microliter of cDNA prepared as described above was amplified in the presence of 1 μL of 5′ and 3′ primers, 0.5 μL of dNTP (Promega), 2.5 μL of *Taq* DNA polymerase 10 × buffer (Promega), and 0.5 μL of *Taq* DNA polymerase (Promega) in a final volume of 25 μL. The

Table 2. Primer sequences used for RT-PCR

Cytokine	Oligonucleotide sequence
IL-1α	5′-AAGATGTCCAACTTCACCTTCAAGGAGAGCCG-3′
	5′-AGGTCGGTCTCACTACCTGTGATGAGTTTTGG-3′
TNF-α	5′-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3′
	5′-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3′
IFN-γ	5′-TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC-3′
	5′-TGGACCTGTGGGTTGTTGACCTCAAACTTGGC-3′
IL-6	5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′
	5′-CACTAGGTTTGCCGAGTAGATCTC-3′
IL-10	5′-CTGGAAGACCAAGGTGTCTAC-3′
	5′-GAGCTGCTGCAGGAATGATGA-3′
β-actin	5′-GTGGGCCGCTCTAGGCACCAA-3′
	5′-CTCTTTGATGTCACGCACGATTTC-3′

PCR reactions were performed in a DNA thermal cycler (Perkin-Elmer-Cetus Instruments, Norwalk, CT). All PCRs started with a 3-min denaturation step that was followed by 35 cycles of 1 min of denaturation at 94 °C, 1 min of annealing temperature, and 1 min of extension at 72 °C. A final 10 min at 72 °C was used in all cases. The annealing temperature used for primers was as follows: IL-1α 60 °C, TNF-α 60 °C, IFN-γ 60 °C, IL-6 60 °C, IL-10 60 °C, and β-actin 60 °C. Twenty-five microliters of the reactions were subjected on 1.5% agarose gel and electrophoresis was performed at 100 V. One microgram of GeneRuler, DNA Ladder Mix (#SM0331, Fermentas), was run in parallel as a molecular weight (MW) marker (providing bands at 100–1000 bp).

Statistics

The immunoenzymatic assays were carried out in triplicate and the results were expressed as the mean ± SD. Comparisons between tests were made by Student's *t*-test, with statistical significance considered to be $P < 0.05$.

Results

Purity of Omp-PA and Bf-OmpA preparations

SDS-PAGE analysis of the purified proteins is shown in Fig. 1. SDS-PAGE of Omp-PA showed one band with a MW of 37 kDa (Fig. 1, Lane 2), as reported previously (Magalashvili *et al.*, 2007). SDS-PAGE analysis of Bf-OmpA demonstrated one band of 37 kDa (Fig. 1, Lane 3), as reported previously (Wexler *et al.*, 2002).

Cloning and expression of *B. fragilis* ompA in *E. coli* and purification of OmpA from inclusion bodies

IPTG-induced *E. coli* BL21 harboring plasmid pET-27b(+)-*ompA* produced inclusion bodies that contained mostly Bf-OmpA (Fig. 2).

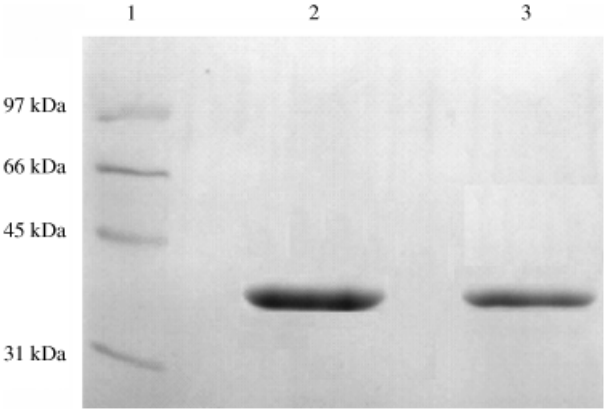

Fig. 1. Electrophoretic pattern of the purified porins from *Porphyromonas asaccharolytica* ATCC 25260 and *Bacteroides fragilis* ATCC 25285. Lane 1, MW standards; lane 2, purified sample of *P. asaccharolytica* porin (37 kDa); lane 3, purified sample of *B. fragilis* porin (36.8 kDa).

Refolding of *B. fragilis* OmpA from inclusion bodies

Densitometric analysis refolded Bf-OmpA indicated that maximal results were achieved with 16 mM zwittergent 3–14 after incubation for 5 days at 37 °C. Approximately 51% of the overexpressed protein could be refolded under these conditions. The refolded Bf-OmpA migrated at the same apparent MW of 36.8 kDa as the native Bf-OmpA (Fig. 2). It was shown earlier that the refolded Bf-OmpA has activity similar to the native protein in the liposome assay (Wexler *et al.*, 2002).

Release of IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 from murine splenocytes induced by Omp-PA from *P. asaccharolytica* and native and recombinant Bf-OmpA from *B. fragilis*

The highest release of the cytokines from murine splenocytes induced by Omp-PA and both native and recombinant Bf-OmpAs was observed after 48 h of incubation. Cells stimulated with 5 μg mL^{-1} Con A were used as positive controls (data not shown), and the nonstimulated cells served as negative controls. LDH levels presented in the supernatants of stimulated cells were similar to those detected in the supernatants of nonstimulated cells, suggesting that cytokine release was not due to cell lysis (data not shown). Omp-PA induced high-level secretion of the proinflammatory cytokines IL-1α, TNF-α, IFN-γ, IL-6, and anti-inflammatory cytokine IL-10 in a dose-dependent fashion and at a concentration ranging from 0.1 to 10 μg mL^{-1}. The amounts (pg mL^{-1}) of each released cytokine are demonstrated in Fig. 3. The highest levels of the cytokine secretion were observed with the Omp-PA concentrations of 0.1 μg mL^{-1} for TNF-α, 1 μg mL^{-1} for IFN-γ and IL-10, and 5 μg mL^{-1} for IL-1α and IL-6. Concentrations higher than

these caused a decrease in cytokine production, and a concentration lower than 0.1 μg mL^{-1} showed no significant effect. Both native and refolded recombinant Bf-OmpAs were able to regulate release of IL-1α, TNF-α, IFN-γ, and IL-6 but in much lower levels compared with those obtained using Omp-PA. Both native and recombinant Bf-OmpAs had no significant effect on the release of IL-10 under the same experimental conditions. The highest levels of the cytokine secretion were observed with the protein concentrations of 0.1 μg mL^{-1} for IL-1α and IFN-γ, 1 μg mL^{-1} for TNF-α, and 5 μg mL^{-1} for IL-6.

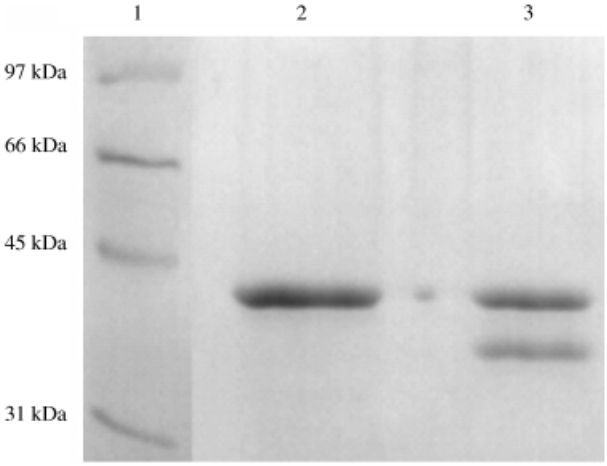

Fig. 2. SDS-PAGE of recombinant Bf-OmpA refolded in zwittergent 3–14. Lane 1, standard proteins; lane 2, recombinant Bf-OmpA recovered from inclusion bodies; lane 3, refolded recombinant Bf-OmpA incubated with 16 mM (3–14) for 5 days at 37 °C.

Cytokine mRNA expression induced by Omp-PA from *P. asaccharolytica* and native and recombinant Bf-OmpA from *B. fragilis*

Expression levels of IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 mRNAs were evaluated by treating murine splenocytes with Omp-PA and native and recombinant Bf-OmpAs in concentrations that showed maximum release of each cytokine as measured by ELISA kits (Fig. 3). The mRNA levels of all five cytokines, IL-1α, TNF-α, IFN-γ, IL-6, and IL-10, were increased upon stimulation of the cells by adding appropriate concentrations of Omp-PA (Fig. 4). The mRNA levels of the cytokines IL-1α, TNF-α, IFN-γ, and IL-6, expressed by stimulation of the cells with native and recombinant Bf-OmpAs, were similarly increased, but were found to be lower compared with those expressed by Omp-PA. Very low expression of IL-10 mRNA was detected under the same experimental conditions. The nonstimulated cells did not show increased mRNA expression of the tested cytokines (Fig. 4).

Discussion

The severity of sepsis (defined as a systemic inflammatory response syndrome associated with infection) is related to the severity of the host response. One measure of this response is the production of cytokines (particularly TNF-α,

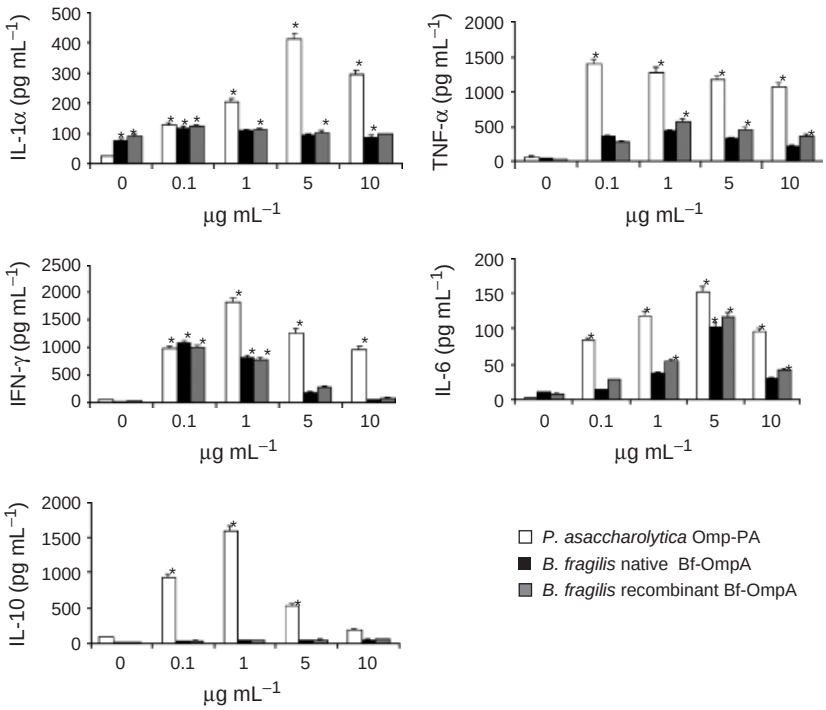

Fig. 3. Release of IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 from murine splenocytes stimulated by *Porphyromonas asaccharolytica* Omp-PA, *Bacteroides fragilis* native Bf-OmpA, and *B. fragilis* recombinant Bf-OmpA at different concentrations after 48 h of incubation. Each point represents the mean of three experiments ± SDs. Points designated by asterisks indicate statistically significant differences ($P < 0.05$) vs. nonstimulated cells.

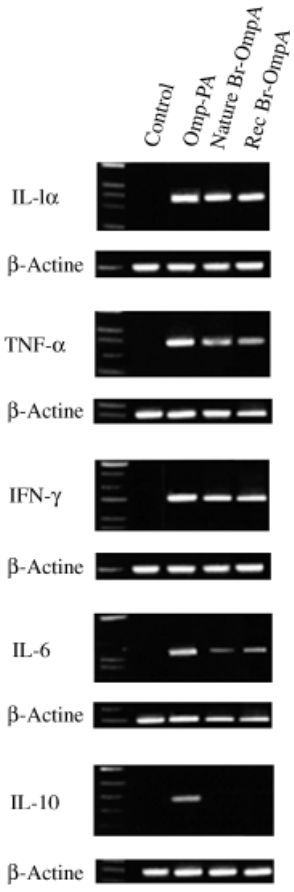

Fig. 4. Cytokine mRNA expression induced by *Porphyromonas asaccharolytica* Omp-PA and *Bacteroides fragilis* native and recombinant Bf-OmpAs in murine splenocytes. Control indicates the nonstimulated cells.

IL-1β, IL-6, and IL-8); although important in host defense functions, these cytokines can also result in widespread tissue injury. The early cytokines, TNF-α and IL-1β, are thought to mediate the production of the later or distal cytokines, including IL-6 and IL-8 (Blackwell & Christman, 1996). Triggering the release of the cytokines TNF-α, IL-1, IL-6, IL-8, and IL-12 and the subsequent inflammatory response is critical to containing bacterial infection in the tissues. If, however, infection disseminates in the blood, the widespread activation of phagocytes in the bloodstream is catastrophic.

Gram-negative bacteria, and specifically the lipopolysaccharide component, are most often implicated in inducing the cytokine cascade. Humans injected with purified lipopolysaccharide develop a cytokine cascade in the serum. The early cytokine response (TNF-α, IL-6, and IL-8) coincides with the onset of fever and the activation of blood neutrophils, monocytes, and lymphocytes.

In this study, we investigated the ability of the OmpA proteins from *P. asaccharolytica* and *B. fragilis* to trigger release and expression of proinflammatory and immunoregulatory cytokines IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 by murine splenocytes *in vitro*. Both native and refolded recombinant *B. fragilis* Bf-OmpA that are proved to be functionally identical (Wexler *et al.*, 2002) could elicit the cytokine release. We were not able to refold the Omp-PA, and therefore, could not test it in this assay. The main reason for using recombinant refolded Bf-OmpA was to avoid the possibility that any capsule contamination in the purified protein preparation may be responsible for the cytokine production (Wexler, 2007). The cytokine release proceeded in a dose-dependent fashion with different concentrations of porins needed for the maximum release of each cytokine. The release of IL-1α and IL-6 stimulated by Omp-PA was relatively low compared with the high-level production of TNF-α, IFN-γ, and IL-10. The results obtained cannot be attributed to the contaminating lipopolysaccharide (10 pg per 10 μg of porin) in the porin preparations, because this trace amount of lipopolysaccharide had no ability to induce any cytokine production (data not shown). Moreover, porins were incubated with polymyxin B to neutralize the biological activity of lipid A (Galdiero *et al.*, 1993). It is proved that the porin–polymyxin complex has the same activity in the induction of the cytokine secretion as the porin preparations alone, while the lipopolysaccharide–polymyxin complex is inactive (Galdiero *et al.*, 1995). While *E. coli* lipopolysaccharide is more active (per μg) than *Bacteroides* lipopolysaccharide in inducing cytokine production, *Bacteroides* strains often outnumber *Enterobacteriaceae* in the feces and in mixed infections, and their role in inducing and/or modulating the host response in septic shock should not be overlooked. Nagy *et al.* (1998) assessed the ability of several species of heat-inactivated *Bacteroides* as well as isolated Lipopolysaccharide to induce TNF-α release and IL-6 production in human mononuclear cells and whole blood. As expected, *E. coli* lipopolysaccharide was more active than *B. fragilis* lipopolysaccharide in inducing these factors; slightly more TNF-α (> 2.5-fold) and more IL-6 (> 10-fold) were produced. Both TNF-α and IL-6 were induced by $10\,\mu g\,mL^{-1}$ Lipopolysaccharide or by $10^9\,CFU\,mL^{-1}$ heat-inactivated cells; we cannot compare the level of induction because their assay used human mononuclear cells and whole blood. In another study, the cytokine induction of *Bacteroides* lipopolysaccharide depended on the extraction method used, and lipopolysaccharide extracted by the phenol–water method had the highest TNF-α-inducing activity (Delahooke *et al.*, 1995a, b). Compared with *E. coli* lipopolysaccharide, the phenol–water extract of *B. fragilis* was only marginally less active (five- to sevenfold) in the bioassay for TNF induction from human mononuclear leukocytes (Delahooke *et al.*, 1995b; Poxton & Edmond, 1995). In *Bacteroides*, only lipopolysaccharide has been studied in terms of ability to induce cytokines, but other cell structures, including porins

or other outer-membrane proteins, have not been investigated (other than this study), although such structures in other organisms are known to act as immune 'modulins' (Henderson *et al.*, 1996). Porins in other organisms such as *Salmonella*, *Yersinia*, and *Pseudomonas* are known to induce cytokines; the number of cytokines induced by *Salmonella* and *Yersinia* porins was in the same general ranges as that induced by PA Omp (Galdiero *et al.*, 1993; Tufano *et al.*, 1994). Direct comparison of inducing ability in *Pseudomonas* (which would be the best analogy because the major porin is an OmpA-like molecule) of the amount of cytokine induced was in Units based on lysis or cell proliferation assays (Cusumano *et al.*, 1997).

Interestingly, Omp-PA was able to induce cytokine release at much higher levels compared with those obtained with native and recombinant Bf-OmpAs. We were initially surprised by this because *P. asaccharolytica* is considered to be less pathogenic than *B. fragilis*. However, mice were used as the experimental animals, and *B. fragilis* is part of mouse intestinal microbial communities. Thus, the mouse was potentially immunized to the Bf-OmpA (Pumbwe *et al.*, 2006), which may explain why the stimulation of murine splenocytes by *B. fragilis* porin did not result in high-level secretion of cytokines.

Alternatively, perhaps other porin proteins present on the *B. fragilis* outer membrane may have more cytokine-stimulating activity. For example, the *B. fragilis* Omp-200 has more pore-forming activity than OmpA (Wexler, 1997). On the other hand, only one active monomeric porin (Omp-PA) was isolated from the outer membrane of *P. asaccharolytica*.

As expected, exposure to Omp-PA and native and recombinant Bf-Omp resulted in increased levels of IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 mRNA expression. The results obtained in reverse transcriptase (RT)-PCR analysis corresponded to those measured by ELISA kits. For comparison purposes, we also checked the ability of another outer-membrane protein, Omp-EA, to induce the release and expression of IL-6 and IFN-γ. Omp-EA is an outer-membrane protein from *Erwinia amylovora*, a pathogen that infects only plants (Elazar *et al.*, 2007). As expected, Omp-EA acted as a negative control and did not induce release of the tested cytokines (data not shown).

Recent reports suggest that the ability to induce cytokine production by the porins is dependent on the existence of the externally exposed loops that have been described extensively (Galdiero *et al.*, 2006). We have recently described these loops in monomeric porins from *Acinetobacter baumannii* (Gribun *et al.*, 2003), *B. fragilis* (Wexler *et al.*, 2002), and *P. asaccharolytica* (Magalashvili *et al.*, 2007). Future work will include studying the role of the external loops of *P. asaccharolytica* Omp-PA and *B. fragilis* Bf-OmpA in induction of cytokine release.

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Authors' contribution

L.M. and S.L. contributed equally to this work.

References

Biswas T (2000) Role of porin of *Shigella dysenteriae* type 1 in modulation of lipopolysaccharide mediated nitric oxide and interleukin-1 release by murine peritoneal macrophages. *FEMS Immunol Med Microbiol* **29**: 129–136.

Blackwell TS & Christman JW (1996) Sepsis and cytokines: current status. *Br J Anaesth* **77**: 110–117.

Blanchard DK, Klein TW, Friedman H & Stewart WE (1986) Induction of gamma interferon by endotoxin by endotoxin in 'aged' murine splenocyte cultures. *Immunobiology and Immunopharmacology of Bacterial Endotoxins* (Sventivanyi A, Friedman H & Nowotny A, eds), pp. 155–163. Plenum Press, New York.

Brinkman FS, Bains M & Hancock RE (2000) The amino terminus of *Pseudomonas aeruginosa* outer membrane protein OprF forms channels in lipid bilayer membranes: correlation with a three-dimensional model. *J Bacteriol* **182**: 5251–5255.

Chomczynski P & Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156–159.

Cusumano V, Tufano MA, Mancuso G, Carbone M, Rossano F, Fera MT, Ciliberti FA, Ruocco E, Merendino RA & Teti G (1997) Porins of *Pseudomonas aeruginosa* induce release of tumor necrosis factor alpha and interleukin-6 by human leukocytes. *Infect Immun* **65**: 1683–1687.

Delahooke DM, Barclay GR & Poxton IR (1995a) Tumor necrosis factor induction by an aqueous phenol-extracted lipopolysaccharide complex from *Bacteroides* species. *Infect Immun* **63**: 840–846.

Delahooke DM, Barclay GR & Poxton IR (1995b) A re-appraisal of the biological activity of *Bacteroides* LPS. *J Med Microbiol* **42**: 102–112.

Elazar M, Halfon D, Pechatnikov I & Nitzan Y (2007) Porin isolated from the outer membrane of *Erwinia amylovora* and its encoding gene. *Curr Microbiol* **54**: 155–161.

Galdiero F, de L'ero GC, Benedetto N, Galdiero M & Tufano MA (1993) Release of cytokines induced by *Salmonella typhimurium* porins. *Infect Immun* **61**: 155–161.

Galdiero M, Cipollaro de l'Ero G, Donnarumma G, Marcatili A & Galdiero F (1995) Interleukin-1 and interleukin-6 gene expression in human monocytes stimulated with *Salmonella typhimurium* porins. *Immunology* **86**: 612–619.

Galdiero S, Vitiello M, Amodeo P, D'Isanto M, Cantisani M, Pedone C & Galdiero M (2006) Structural requirements for proinflammatory activity of porin P2 loop 7 from *Haemophilus influenzae*. *Biochemistry* **45**: 4491–4501.

Gribun A, Nitzan Y, Pechatnikov I, Hershkovits G & Katcoff DJ (2003) Molecular and structural characterization of the HMP-AB gene encoding a pore-forming protein from a clinical isolate of *Acinetobacter baumannii*. *Curr Microbiol* **47**: 434–443.

Gupta S (1998) Priming of T-cell responses in mice by porins of *Salmonella typhimurium*. *Scand J Immunol* **48**: 136–143.

Henderson B, Poole S & Wilson M (1996) Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol Rev* **60**: 316–341.

Huang SH, Stins MF & Kim KS (2000) Bacterial penetration across the blood-brain barrier during the development of neonatal meningitis. *Microbes Infect* **2**: 1237–1244.

Iovane G, Pagnini P, Galdiero M, Cipollaro de l'Ero G, Vitiello M, D'Isanto M & Marcatili A (1998) Role of *Pasteurella multocida* porin on cytokine expression and release by murine splenocytes. *Vet Immunol Immunopathol* **66**: 391–404.

Jousimies-Somer HR, Summanen P, Citron DM, Baron EJ, Wexler HM & Finegold SM (2002) Advanced identification methods. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th edn. (Finegold SM & Jousimies-Somer HR, eds), pp. 81–132. Star Publishing Company, Belmont, CA.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **277**: 680–685.

Magalashvili L, Pechatnikov I, Wexler HM & Nitzan Y (2007) Isolation and characterization of the Omp-PA porin from *Porphyromonas asaccharolytica*, determination of the *omp-PA* gene sequence and prediction of Omp-PA protein structure. *Anaerobe* **13**: 74–82.

Melon O, Olles JM, Barreiro A, Verdugo A, Mendez FJ & Vasquez F (1997) Bacteremia by *Porphyromonas asaccharolytica*. *Enferm Infecc Microbiol Clin* **15**: 121 (in Spanish).

Nagy E, Mandi Y, Szoke I & Kocsis B (1998) Induction of release of tumor necrosis factor and IL-6 from human mononuclear cells by Bacteroides strains. *Anaerobe* **4**: 133–138.

Nitzan Y, Pechatnikov I, Bar-El D & Wexler H (1999) Isolation and characterization of heat-modifiable proteins from the outer membrane of *Porphyromonas asaccharolytica* and *Acinetobacter baumannii*. *Anaerobe* **5**: 43–50.

Ofori-Darko E, Zavros Y, Rieder G, Tarle SA, Van Antwerp M & Merchant JL (2000) An OmpA-like protein from Acinetobacter spp. stimulates gastrin and interleukin-8 promoters. *Infect Immun* **68**: 3657–3666.

Perfetto B, Donnarumma G, Criscuolo D, Paoletti I, Grimaldi E, Tufano MA & Baroni A (2003) Bacterial components induce cytokine and intercellular adhesion molecules-1 and activate transcription factors in dermal fibroblasts. *Res Microbiol* **154**: 337–344.

Poxton IR & Edmond DM (1995) Biological activity of *Bacteroides* lipopolysaccharide – reappraisal. *Clin Infect Dis* **20** (suppl 2), S149–S153.

Prasadarao NV, Wass CA, Weiser JN, Stins MF, Huang SH & Kim KS (1996) Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infect Immun* **64**: 146–153.

Prasadarao NV, Wass CA, Stins MF, Shimada H & Kim KS (1999) Outer membrane protein A-promoted actin condensation of brain microvascular endothelial cells is required for *Escherichia coli* invasion. *Infect Immun* **67**: 5775–5783.

Pumbwe L, Skilbeck CA & Wexler HM (2006) The *Bacteroides fragilis* cell envelope: quarterback, linebacker, coach-or all three? *Anaerobe* **12**: 211–220.

Revankar SG & Clark RA (1998) Infected cardiac myxoma. Case report and literature review. *Medicine (Baltimore)* **77**: 337–344.

Tufano MA, Rossano F, Catalanotti P, Liguori G, Marinelli A, Baroni A & Marinelli P (1994) Properties of *Yersinia enterocolitica* porins: interference with biological functions of phagocytes, nitric oxide production and selective cytokine release. *Res Microbiol* **145**: 297–307.

Wexler HM (1997) Pore-forming molecules in gram-negative anaerobic bacteria. *Clin Infect Dis* **25** (suppl 2), S284–S286.

Wexler HM (2007) Bacteroides: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev* **20**: 593–621.

Wexler HM, Read EK & Tomzynski TJ (2002) Identification of an OmpA protein from *Bacteroides fragilis*: ompA gene sequence, OmpA amino acid sequence and predictions of protein structure. *Anaerobe* **8**: 180–191.

Yin ET, Galanos C, Kinsky S, Bradshaw RA, Wessler S, Luderitz O & Sarmiento ME (1972) Picogram-sensitive assay for endotoxin: gelation of *Limulus polyphemus* blood cell lysate induced by purified lipopolysaccharides and lipid A from Gram-negative bacteria. *Biochim Biophys Acta* **261**: 284–289.

Characteristics of *Bacteroides fragilis* lacking the major outer membrane protein, OmpA

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Running title: *Bacteroides fragilis* OmpA protein

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Summary

OmpA1 is the major outer membrane protein of the gram-negative anaerobic pathogen, *Bacteroides fragilis*. We identified three additional conserved *ompA* homologs *(ompA2-ompA4*) and three less homologous *ompA*-like genes *(ompA*s *5,6 and 7*) in *B. fragilis*. We constructed an *ompA1* disruption mutant in *B. fragilis* 638R (WAL67Ω*ompA1*) using insertion-mediated mutagenesis. WAL67Ω*ompA1* formed much smaller colonies and had smaller, rounder forms on gram stain analysis than the parental strain or other unrelated disruption mutants. SDS-PAGE and Western Blot analysis (with anti-OmpA1 IgY) of the OMP patterns of WAL67Ω*ompA1* grown in both high and low salt media did not reveal any other OmpA proteins even under osmotic stress. An *ompA1* deletant (WAL186Δ*ompA1*) was constructed using a two-step double-crossover technique and an *ompA* "reinsertant" WAL360+*ompA1* was constructed by reinserting the *ompA* gene into WAL186Δ*ompA1*. WAL186Δ*ompA1* was significantly more sensitive to exposure to SDS, high salt and oxygen than the parental (WAL 108) or reinsertant (WAL360+*ompA1*) strain. No significant change was seen in MICs of a variety of antimicrobials for either WAL67Ω*ompA1* or WAL186Δ*ompA1* compared to WAL108. RT-PCR revealed that all of the *ompA* genes are transcribed in the parental strain and in the disruption mutant, but, as expected, *ompA1* is not transcribed in WAL186Δ*ompA1*. Unexpectedly, *ompA4* is also not transcribed in WAL186Δ*ompA1*. A predicted structure indicated that among the four OmpA homologs, the barrel portion is more conserved than the loops, except for specific conserved patches on loop 1 and loop 3. The presence of multiple copies of such similar genes in one organism would suggest a critical role for this protein in *B. fragilis*.

INTRODUCTION

Bacteroides fragilis is a major component of the gastrointestinal flora and the most frequent anaerobic pathogen (Finegold & Wexler, 1996). Until the publication of the genome sequence in 2004-5 (Cerdeno-Tarraga *et al.*, 2005b;Cerdeno-Tarraga *et al.*, 2005a;Kuwahara *et al.*, 2004), only a few genes coding for outer membrane proteins (OMPs) in any of the species of *Bacteroides* had been identified. The subsequent publication of the genome sequence revealed that *B. fragilis* has dozens of genes coding for OMPs and only a fraction of those have been characterized. The *B. fragilis* OMPs that have been most studied are those involved in capsule formation and porin function (Comstock *et al.*, 1999;Cheng *et al.*, 1995;Reeves *et al.*, 1996;Reeves *et al.*, 1997;Shipman *et al.*, 2000;Wexler *et al.*, 2002a;Wexler *et al.*, 2002b;Kanazawa *et al.*, 1995;Odou *et al.*, 1998;Odou *et al.*, 2001). Significant work has been accomplished on the genes involved in capsule formation, their regulation and their importance in immune modulation and host colonization (Comstock & Kasper, 2006;Cassel *et al.*, 2008;Coyne *et al.*, 2008;Liu *et al.*, 2008). However, most of the work on other membrane proteins and the associated phenotypes were done years before the genome sequence was done, and consequently, the phenotypic traits already described cannot be assigned to a particular gene product.

The *B. fragilis omp*A1 gene encodes a homolog of a major outer membrane protein gene that is fairly ubiquitous in bacteria (Wexler et al., 2002b); the examples most studied are *E. coli* OmpA and *Pseudomonas* OprF. Among anaerobic bacteria, *ompA* homologs have been well studied in *Porphyromonas gingivalis* (Yoshimura *et al.*, 2008;Iwami *et al.*, 2007;Nagano *et al.*, 2005;Imai *et al.*, 2005;Murakami *et al.*, 2002), formerly a member of the genus *Bacteroides* and a prominent pathogen in adult periodontal diseases (Mineoka *et al.*, 2008). OmpA proteins are among the most conserved of all OMPs in bacteria and consist of an outer membrane spanning β-barrel domain composed of eight β-sheets and a peptidoglycan-linked periplasmic domain. OmpA has been implicated in maintaining cell structure, biofilm formation, adhesin, invasin and evasin properties, colicin and bacteriophage receptors (Smith *et al.*, 2007), macrophage activation (Soulas *et al.*, 2000;Wang & Kim, 2002;Wang & Kim, 2002;Prasadarao *et al.*, 1996) and virulence (Huang *et al.*,

2000). Although OmpA1 is the major OMP of *B. fragilis*, its function in this genus has not been described. The purpose of this work was to study the function of the OmpA1 protein in *B. fragilis* by constructing and characterizing strains with defective or absent OmpA1 and comparing them with the parental strain.

Materials and Methods:

Growth of *B. fragilis*. The bacterial strains, plasmids and primers used are listed in Table 1. *B. fragilis 638R* is a clinical isolate and is the strain generally used in research laboratories for genetic manipulations. The published *B. fragilis* sequence is that of the *B. fragilis* NCTC 9343 (Cerdeno-Tarraga et al., 2005b;Cerdeno-Tarraga et al., 2005a;Kuwahara et al., 2004). Sequencing data for *B. fragilis* 638R were produced by the *Bacteroides fragilis* Sequencing Group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/bf/BF638R.dbs. *B. fragilis* 638R and pFD516 (a gift of Dr. C. J. Smith, East Carolina University, Greenville, NC) were used for construction of the disruptant WAL67Ω*ompA*. *B. fragilis* ADB77 (a thy deficient mutant of *B. fragilis 638R* and named WAL108 in our laboratory) and the suicide plasmids pYT102 and pADB242b (used for construction of the deletant and reinsertant, respectively) were a kind gift from Dr. Michael Malamy (Tufts University, Boston, MA). Strains were grown as described previously (Pumbwe *et al.*, 2005) in brain heart infusion broth supplemented with thymine (Baughn & Malamy, 2002) or in anaerobic minimal medium with 0.5% glucose (AMMgluc) (Baughn & Malamy, 2002). Thymine (50 µg/mL) was added for growth of thy^- strains (i.e., WAL 108 and its derivatives).

Strains and constructs are named with the WAL designation and the genotype in respect to the *ompA1* gene: *B. fragilis* 638R, WAL108 (parental), WAL67Ω*ompA1* (*ompA1* disruptant), WAL186Δ*ompA1* (*ompA1* deletant), WAL360+*ompA1* (*ompA1* reinsertant). Construction of the strains is described below.

Determination of MIC. MICs were measured by the Spiral Gradient Endpoint (SGE) method (MIC) (Wexler *et al.*, 1996;Wexler, 1991) on at least three independent occasions.

Construction of WAL67Ω*ompA1* (*ompA1* disruption mutant). A fragment containing an internal portion of the predicted β-barrel encoding region of *ompA1* was amplified from *B. fragilils* 638R and cloned into pCR2.1 (TOPO™ TA Cloning® Kit, Invitrogen,Carlsbad, CA). The gene fragment was excised by

EcoRI digestion and ligated into the *ecoR1* site of pFD516. *E. coli* Top10F was transformed with pFD516::'*ompA1*' and plasmids were prepared with the Qiagen MiniPrep Kit (Qiagen). The gel-purified plasmids were used to transform *E. coli* HB101 by electroporation and transformants were maintained by selection with chloramphenicol. Tri-parental matings with *E. coli* HB101/pFD516:: '*ompA1*', *E. coli* DH5α/pRK231 (a mobilizing plasmid) and *B. fragilis* 638R were conducted. Transconjugants were selected on BHIS plates containing gentamicin (Gen), rifampin (Rif) (to select against the donor) and erythromycin (Erm) (to select for recombinants). Disruption mutants were confirmed by PCR and sequencing and maintained in media with 10 ug ml^{-1} of erythromycin. The disruption mutant was named WAL67Ω*ompA1*.

Construction of WAL186 Δ*ompA1* (*ompA1* deletion mutant). An in-frame deletion of *ompA1* was constructed by a two-step double cross-over technique with pYT102 (Baughn & Malamy, 2002). Briefly, 800 bp fragments of the upstream and downstream regions (including approximately 50-100 bp of the beginning and end of *ompA1*) were amplified using specific primers to which appropriate restriction sites were added for subsequent cloning into pYT102 (Table 1). pYT102 was digested with BamH1 and HindIII and gel purified. PCR amplicons were digested with BamH1/NCO1, or HindIII/NCO, respectively, and mixed with BamH1/HindIII digested pYT102 in a three part ligation as described (Pumbwe *et al.*, 2006).

Chemically competent *E. coli* DH5α was transformed with pYT102::'*ompA1updown*' and transformants selected by chloramphenicol. pYT102::'*ompA1updown*' was mobilized into *B. fragilis ADB77* in a three part mating with *E. coli* DH5α/ pYT102::'*ompA1updown*' and *E. coli* HB101/pRK231 (Baughn & Malamy, 2002). Cointegrants were selected by gentamicin (50 µg/ml), rifampicin (50 µg/ml) and tetracycline (2 µg/ml), confirmed by colony PCR using primers designed to detect the recombinant junction and maintained on media with tetracycline. The second step recombination was done as described (Baughn & Malamy, 2002). Trimethoprim resistant colonies were screened to confirm that they were tetracycline sensitive, and further screened by PCR with sets of both internal and junction primers to confirm that they were the desired deletion resolution products. Deleted genes were verified by DNA sequencing of the deletion junction. The *ompA1* deletant was named WAL186Δ*ompA1*.

Construction of WAL360 + *ompA1* (i.e., *ompA1* reinsertant). The full-length *B. fragilis ompA1* gene (including about 800 base pairs upstream and downstream of the gene) was cloned in the suicide vector pADB242b. The recombinant plasmid was verified by DNA sequencing. *E.coli DH5α*/pADB242b-up*ompA1*down and *E.coli DH5α* /*pRK231* were mated with *B. fragilis* WAL186Δ*ompA1* as described (Baughn & Malamy, 2002) and the cointegrants selected as described above. Cointegrants were plated on minimal media with thymine and trimethoprim to select for the second recombination event. Reinsertants containing full length *ompA1* were confirmed by sequencing and the *ompA* "reinsertant" was named WAL360+*ompA1*.

Cloning and expression of *B. fragilis ompA1* in *E. coli*, purification of OmpA1 from inclusion bodies, and production of anti-OmpA1 IgY. Recombinant OmpA1 was prepared as described (Wexler et al., 2002b). Briefly, *B. fragilis ompA1* was cloned into pET- 27b(+) (Novagen, Madison, WI). Purified plasmid DNA was used to transform BL21 (DE3) pLysS (Novagen) according to the manufacturer's instructions. Cells were grown and inclusion bodies prepared according to manufacturer's directions. Gel-purified OmpA1 was submitted to Aves Lab (Tigard, OR) for production of anti-OmpA1 IgY.

Cells from a 500 ml overnight culture were harvested by centrifugation at 6000 x g for 20 min in a Sorvall RC-5B centrifuge, and washed once in 10 mM Tris-HCl containing 10 mM $MgSO_4$. Cells were broken by four passages through a French pressure Cell (SLM Instruments, Urbana, IL, USA) at 12000 lb/in^2. The suspension was centrifuged at 6000 x g for 5 min to remove whole cells and cell debris. The supernatant contained the cell envelopes and cytoplasm. The inner membrane was solubilized by adding 2% Triton X-100 containing 10 mM $MgCl_2$ and 10 mM HEPES (final concentration) to the supernatant and incubating for 30 min at room temperature, and then centrifuging at 45000 x g (1 hour). The resulting pellet containing the crude outer membrane (OM) was washed once with 10 mM Tris-HCl, 10 mM $MgSO_4$ pH 7.4 and then frozen.

SDS-PAGE and Western Blot analysis of *B. fragilis* outer membrane preparations. Outer membranes were prepared as described (Wexler et al., 2002b). SDS-PAGE was performed as described (Gallagher, 1987) using a modified Laemmli gel (Laemmli & Favre, 1973). Samples were incubated in SDS sample buffer at either room temperature or at 100 ^oC for 5 minutes. Molecular weights were based on

molecular weight standards (Sigma, St. Louis, Mo.). The gel was stained by a rapid technique and destained extensively with several changes of destain buffer (Wong *et al.*, 2000). The Western Blot was performed as described (Ausubel *et al.*, 1987) using a 1:5000 dilution of the anti-OmpA1 IgY.

RNA extraction. Total cellular RNA was isolated from strains cultured in BHI broth containing 5% thymine using the RNeasy-RNA Protect™ (Qiagen; Valencia, CA) method with on-column DNase treatment. Strains were incubated for 2 hours under anaerobic conditions to a mid-log phase of growth (OD600 = 0.4). Aliquots (3mL) were mixed with an equal volume of RNA-Protect™ and the extraction continued according to manufacturer's instructions. A standard PCR confirmed that the RNA was free of chromosomal DNA contamination. The integrity of the extracted RNA was confirmed by agarose gel electrophoresis and by spectrophotometric analysis ($OD_{260/280}$). Samples were quantified by OD_{260} measurement and the measurement converted to ng/ µl.

Reverse transcriptase PCR. DNA-free total RNA was isolated from *B. fragilis* 638R using the RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Primer pairs specific for each of the *B. fragilis ompA* gene homologs (Table 1) and total *B. fragilis* RNA (10 ng per reaction) were used in the OneStep RT-PCR Kit (QIAGEN, Valencia, CA) to amplify specific transcripts according to the manufacturer's instructions. Amplification products were separated on a 1.5% (w/v) agarose-TAE gel containing 5 g/ml^{-1} ethidium bromide.

Quantification of gene expression by quantitative comparative real time RT-PCR. Briefly, two-step real-time PCR was performed with the Cepheid SmartCycler® using the Quantitect® SYBR® Green one-step RT-PCR kit (Qiagen). Primers were designed to amplify products between 130-170 bp in size and were added to the reactions at a final concentration of 1.0 µM each. RNA samples were added to the reactions to result in 200 ng/reaction, except for the 16SRNA samples that were added to a final amount of 200 pg/reaction. Expression levels were measured as an amount of cDNA as extrapolated by a cycle threshold (Ct) value from the standard real time PCR growth curve. The Ct was the cycle number at which the growth curve attained exponential growth and was thus the highest concentration of template. In order to rule out any non-specific products resulting from primer-dimers, melting curve analysis of the amplified products was

performed. RNA expression was normalized to the parental strain by using 16SRNA. Expression results were quantified by the comparative cycle threshold approximation method (Stintzi *et al.*, 2005), using the assumption that the PCR growth curve efficiency for all reactions is 100% and that the DNA concentration doubled at each cycle:

ΔΔCt (fold-change in expression) = 2(Ctparental - Ct16srRNA) – 2(Ctdeletant- Ct16srRNA)

Data were analyzed by Student's T test and a value of $P < 0.05$ was considered significant. A ≥2-fold change in expression compared to the parental strain was considered significant.

SDS, acid and high salt sensitivity assays. Challenge with SDS, acid, and high salt were performed as described by Wang for *E. coli* OmpA (Wang, 2002) using media and incubation conditions appropriate for *B. fragilis*. Bacteria were grown in BHIS broth to an OD_{600} 0f 0.6 and diluted in 0.154 M NaCl (equivalent to physiological saline-- 0.9% NaCl) to 10^4 CFU/ml, and plated on BHIS agar containing various concentrations of SDS. Plates were incubated anaerobically for 48 h at 37°C, and CFU were counted. For acid survival, the exponential phase bacteria were diluted 30-fold in PBS. 1/10 volume of the suspension was mixed with BHIS containing acetic acid to a final pH of 3.8, and incubated at 37°C for 20 minutes. Plates were incubated anaerobically for 48 h at 37°C, and CFU were counted. For the high osmolarity challenge, a 1:30 bacterial suspension was mixed with an equal volume of either 0.154 M or 5M NaCl and incubated at room temperature for 2 h. Plates of varying dilutions were incubated anaerobically for 48 h at 37°C, and CFU were counted.

Oxygen sensitivity assay. The oxygen sensitivity of the *ompA* deletant was measured in an agar tube assay (Rocha *et al.*, 2007). Strains were grown in BHIS/thy anaerobically at 37°C. One hundred microliters of overnight (stationary phase) cultures were mixed with 5 ml of BHIS/thy and 0.4% agar in a clear polystyrene tube and incubated aerobically at 37°C for 48 hours. The distance between the top of the agar and the visible growth within the agar was measured.

Genomic and proteomic analyses. The signal sequence cleavage site was predicted by SignalP V2.0. SignalP is comprised of two signal peptide prediction methods, SignalP-NN (based on neural networks) and SignalP-HMM (based on hidden Markov models) (Nielsen *et al.*, 1997). The PSORT

algorithm analyzes a submitted sequence for signal sequences, cleavage sites, amino acid composition, and potential transmembrane regions and then predicts the subcellular location of the protein being analyzed. CLUSTALW1.8 (http://www.ebi.ac.uk/clustalw) (Thompson *et al.*, 1994) was used to generate the alignments.

β-sheet prediction and model prediction. The prediction of β-sheets was kindly done by Dr. Tilman Schirmer (University of Basil) according to his published method (Schirmer & Cowan, 1993). The multiple alignment based on ClustalW was threaded onto the *E. coli* OmpA crystal structure and regions of conservation analyzed by surface-mapping of phylogenetic information using the program CONSURF (Landau *et al.*, 2005;Glaser *et al.*, 2003) and visualized with the Protein Explorer program (http://www.umass.edu/microbio/chime/pe_beta/pe/protexpl/).

RESULTS

Identification of *ompA* homologs in *B. fragilis*. In studies completed before the publication of the *B. fragilis* genome sequence, we identified the major outer membrane protein gene (*ompA1*) in *B. fragilis* (Wexler et al., 2002b) and subsequently identified three additional *ompA* genes using a TBLASTN search against genomic data from the *B. fragilis* sequence data (http://www.sanger.ac.uk/Projects/B_fragilis/). The amino acid sequences for OmpA1, OmpA2 and OmpA4 are identical for *B. fragilis* strains ATCC 25285 and *B. fragilis* 638R. There are two amino acid differences between *B. fragilis* 25285 OmpA3 and *B. fragilis* 638R OmpA3 (F19 →L, K225 →R). According to our model, F19 is in the leader sequence, before the cleavage site and K225 is in the periplasmic portion, just after the last β-strand, thus the barrel portion of the four OmpA homologs is completely conserved in these two strains. Subsequent analysis of the annotated *B. fragilis* sequence revealed three additional *ompA* family homologs (OmpAs 5, 6 and 7) that are somewhat removed from the OmpA1-4 cluster but do contain the OmpA signature domain at the C-terminal (http://expasy.org/prosite/PDOC00819). OmpA5 is approximately the same lengths as OmpAs1-4 (372-399 amino acids); OmpA6 has 224 amino acids and OmpA7 has 616 amino acids. OmpAs 1-7 correspond to *B. fragilis* NCTC 9343 (ATCC 25285) genes BF 3810, 1988, 1689, 1959, 3801 and 1285, respectively.

Conservation of *B. fragilis* OmpAs and homology to OmpAs from other organisms. MUSCLE (http://bioinfo.genotoul.fr/multalin/multalin.html) (Multiple Sequence Comparison by Log Expectation) was used to generate a phylogram that includes *B. fragilis* OmpAs 1-7, *P. gingivalis* 42 kDa antigen (OmpA-like), *Pseudomonas marginalis* OprF and *E. coli* OmpA (Figure 1). *B. fragilis* OmpAs 1-4 exhibit considerable homology throughout the protein sequence (Expect values of OmpAs 2, 3, and 4 in respect to OmpA1 are $2e^{-48}$, $1e^{-46}$, $3e^{-41}$, respectively, with homology across the entire length of the protein, and particularly in the predicted beta strands). OmpAs 2 and 4 are the most homologous (Identities = 331/373 (88%), Positives = 351/373). OmpAs 5 and 6 show less homology (Expect values $7e^{-10}$ and $9e^{-10,}$ respectively, compared to OmpA1) and OmpA7 shares some homology in the beta strand region. All seven homologs share the characteristic C terminal OmpA domain (data not shown). A cladogram including seven *B. fragilis* OmpAs, *P. gingivalis* 42 kDa antigen, and the OmpAs homologs in *E. coli* and *Pseudomonas*, respectively, is shown in Figure 1.

Characterization of WAL67Ω*ompA1*. Amplification and sequencing of the recombination junction verified the disruption of the *ompA1* gene in WAL67Ω*ompA1*. The absence of the OmpA1 protein in WAL67Ω*ompA1* was confirmed by SDS-PAGE analysis of the outer membrane and Western Blot analysis conducted with anti-OmpA1 IgY antisera (data not shown). WAL67Ω*ompA1* grew much more slowly than both *B. fragilis* 638R and other unrelated *omp* mutants constructed with the pFD516 suicide vector at the same time. After 48 hours, only pinpoint colonies were seen, compared to robust 1- 2 mm colonies for the other disruptants and the wild-type strain. Examination of gram stains revealed that WAL67Ω*ompA1* were shorter and rounder than the other strains. The geometric mean cell length for BF638R and WAL67Ω*ompA1* were, respectively: 638 parental: 1.5 μ, and WAL67Ω*ompA1*: 0.79 μ; strains carrying disruptions in *nanH* or other unrelated *omp* genes did not show similar changes.

OmpA homologs were not induced in WAL67Ω*ompA1* under conditions of osmotic stress. Since OmpA and OprF are important for stabilizing cells in hypoosmolar media in *E. coli* and *P. aeruginosa*, respectively, we grew *B. fragilis*638R and WAL67Ω*ompA1* in normal and low salt conditions to see if

perhaps one of the other *ompA*s would be expressed in the mutant under a stressed condition. Cells were grown in salt-free medium with and without salt added (200 mM NaCl) and outer membranes were analyzed by SDS-PAGE and Western Blot with anti-OmpA IgY antisera. No OmpA-like proteins were seen in the mutant, regardless of the salt concentration in the growth medium (data not shown). It is possible that they are expressed at such a low level that we could not detect them in SDS-PAGE and/or that the anti-OmpA1 IgY did not recognize them, despite the considerable homology in the beta strand region among the homologs (since the antisera was prepared using gel-purified OmpA1 as antigen, antibodies to all of the regions, including beta-strands, would be expected to be present in a polyclonal antiserum).

Construction and characterization of WAL186Δ*ompA1*. Sequence analysis confirmed the deletion of the *ompA1* gene and SDS PAGE analysis confirmed the lack of the OmpA1 protein in WAL186Δ*ompA1* (Figure 2). No other OmpAs were detected in WAL186 WAL186Δ*ompA1* using silver stain analysis (data not shown). We believe that although *ompAs* 2, 3, and 4 are transcribed in WAL108 (and *ompAs* 2 and 3 in WAL186Δ*ompA1*), OmpAs 2, 3 and 4 are not expressed in sufficient levels to be detected in the SDS-PAGE analysis of the outer membrane preparation. Colonies of WAL186Δ*ompA1* were much smaller than those of WAL108 (parental) or WAL360+*ompA*1; these results echoed those seen with WAL67*ΩompA1*.

Resistance of WAL108 and WAL186Δ*ompA1* to osmotic stress. WAL 186Δ*ompA1* was more sensitive than WAL 108 to exposure to both SDS and high salt. Exposure of WAL108 to 5M NaCl for 2 hours resulted in a 3 $\log_{10}$ reduction in growth ($1x10^8$ to $5x10^5$); WAL186Δ*ompA1* did not grow at all after exposure to high salt. Similarly, growth of WAL108 on media containing 0.05-0.2% SDS resulted in a 3 $\log_{10}$ reduction in growth as compared to growth on media without SDS ($1x10^8$ to 5.3, 5, and $4.5x10^5$ on 0.05%, 0.1% and 0.2% SDS, respectively); WAL186Δ*ompA1* did not grow at all on media containing even 0.05% SDS. No change in growth between WAL108 and WAL186Δ*ompA1* was seen after exposure to low pH.

Transcription of *ompA* homologs in *B. fragilis* 638R, WAL108, WAL67 Ω*ompA1* , and WAL186Δ*ompA1*. Transcription levels of the *ompA* homologs in the *B. fragilis* constructs are shown in

Table 2. The major transcribed homolog is *ompA1*, followed by *ompA3*, *ompA2* and *ompA4*, respectively. Our studies with WAL67Ω*ompA* had already indicated that *B. fragilis* OmpA1 is important in maintaining cell structure; therefore, we initially assumed that the organism might compensate for the loss of *ompA1* by increasing transcription of one of the other *ompA* homologs. However, we found that transcription of *ompA4* is significantly reduced in WAL186Δ*ompA1*, suggesting the presence of a positive regulatory mechanism to control *ompA4* transcription that is dependant on *ompA1*. Interestingly, the same effect is not seen in WAL67Ω*ompA*. We speculated that perhaps the truncated *ompA1* gene or gene product in the disruption mutant can fulfill the function of the full length product in regulating *ompA4* transcription.

Response of *ompA* transcription levels to high salt. Exposure of WAL 108 and WAL186Δ*ompA1* to 200mM NaCl significantly reduces transcription of all four *ompA* homologs in WAL108 and of *ompAs 2, 3,* and *4* in WAL186Δ*ompA1* (Table 2). Gram stain analysis indicated similar morphology in both WAL108 and WAL186Δ*ompA1* grown on normal media (somewhat pleomorphic gram-negative rods). Gram stain analysis of the strains grown overnight with 200 mM NaCl added revealed that both WAL108 and WAL186Δ*ompA1* assumed very small, round forms.

***B. fragilis* WAL186Δ*ompA1* is more sensitive to oxygen than WAL 108.** WAL186Δ*ompA1* was more sensitive to oxygen stress than either WAL108 or WAL360+*ompA1* (the *ompA* reinsertant), indicating that the absence of the *ompA1* gene, and not some downstream effect or other random mutation, was responsible for the change in sensitivity to oxygen (Figure 3)**.** The average measurements between the top of the agar and the visible growth within the agar were: *B. fragilis* 638R, 6.8 mm; WAL108, 9.2 mm; WAL186Δ*ompA1*, 14 mm; and WAL360+*ompA1*, 9.8 mm. Incubation for an additional 24 hours did not affect the results.

***B. fragilis* OmpA1 does not appear to be important in transport of antimicrobials into the cells.** Susceptibility testing was performed for a wide variety of antimicrobials including β-lactams (ampicillin, cefoperazone, cefoxitin, cephalexin, ceftizoxime), carbapenems (doripenem, ertapenem, faropenem, imipenem, meropenem), quinolones (ciprofloxacin, gatifloxacin, norfloxacin, levofloxacin,

moxifloxacin), chloramphenicol, metronidazole, clindamycin, erythromycin and tetracycline. No significant change was seen in MICs for WAL 108 and WAL186Δ*ompA1*.

Nucleotide sequences and genetic organization of *B. fragilis ompAs*. Potential promoters upstream of the start codon for *B. fragilis ompAs* were identified based on the consensus promoter sequences (Bayley *et al.*, 2000)**.** *ompA2* and *ompA4*, which share the most homology of the four genes, are separated by ~1000 bp, are in inverse orientation and may be the result of a duplication event. Both genes have very conserved upstream sequences that have low level homology (Expect level $1e^{-5}$) to *Vibrio cholera otnG* (involved in cell wall polysaccharide biosynthesis.) Also, pairwise BLASTN of the upstream sequences revealed a highly conserved 200 bp region (Expect level $5e^{-50}$) upstream of the *otnG*-like sequences.

Amino acid sequences and signal peptide sequence of *B. fragilis* OmpAs. The homology of OmpAs 1-4 extends throughout the entire ORF with 30% to 34% identity and 49% to 50% similarity. OmpA2 and OmpA4 are the most homologous pair (84% identity and 89% similarity). The homology is more marked in the carboxy-terminal region and all have significant and comparable homology with the conserved domain database entry for the OmpA family. Both in *E. coli* OmpA and *Pseudomonas* OprF, the N-terminal transmembrane domain and the C-terminal periplasmic region are connected by a hinge region (Chen *et al.*, 1980) composed of an alanine-proline (A-P) repeat preceded by a phenylalanine a few residues earlier (Vogel & Jahnig, 1986) (Woodruff & Hancock, 1989) . While no A-P repeat was seen in *B. fragilis* OmpA, there is an arginine-proline-methionine-proline (RPMP) segment, preceded by two phenylalanines (2 and 6 bases earlier). This segment may serve the same function as the OmpA hinge. When we examined the sequence just before the potential RPMP hinge in the *B. fragilis* OmpAs 1-4s, we found striking similarity to the corresponding regions in *E. coli* and *Shigella* OmpAs (Figure 4). *B. fragilis* OmpAs 1-4 sequences have a "terminal" phenylalanine as the last amino acid of the last β-sheet of the β-barrel, which is considered essential in many OMPs (Struyve *et al.*, 1991) and is consistent with OmpAs from other species. In addition, OmpAs1-4 homologs have hydrophobic amino acids at positions -3, -5, -7 and –9 relative to the terminal phenylalanine that is also characteristic of porin proteins. The corresponding region in *Pseudomonas* is different, although completely conserved in four different species. Alignment of this region of *B. fragilis*

OmpA1-4 with the last β-sheet of *E. coli* OmpA was helpful in constructing the alignments used in the structural predictions for *B. fragilis* OmpA. The carboxy-terminal 17 to 27 amino acids of the *B. fragilis* OmpA homologs do not align with the OmpA-domain consensus and may reflect the phylogenetic distance of *B. fragilis* from the constituents that define the OmpA family.

Signal sequences and cleavage sites for each of the homologs predicted by both SignalP and Psort were in agreement. OmpA1, OmpA2, and OmpA4 have the typical A-X-A sequence preceding the cleavage site (von Heijne, 1985). Other residues are found, often in conjunction with an alanine, as is seen with OmpA3 (VFA). Also, the bulky aromatic residue at the X position (i.e., phenylalanine) often present (von Heijne, 1983) is seen in OmpA3. In addition, the PSORT algorithm predicts, with varying degrees of certainty, outer membrane localization of each of the homologs. Interestingly, the algorithm also affirms, at a lower level of certainty, a periplasmic localization for the *B. fragilis*, as well as the *E. coli*, OmpAs, presumably a reflection of the periplasmic OmpA domain.

Proposed secondary structure of *B. fragilis* OmpAs. *B. fragilis* OmpAs 1-4 were submitted for 3D-PSSM fold recognition analysis (a method using 1D and 3D sequence profiles coupled with secondary structure and solvation potential information potential information (Kelley *et al.*, 2000)); the predicted structures of all four homologs were transmembrane β-barrels. Dr. Tilman Schirmer (University of Basil) kindly analyzed these proteins for the presence of β-sheets (Schirmer & Cowan, 1993). Using the multiple alignment of these sequences, rather than a single sequence, proved to be very helpful in predicting secondary structure for these proteins. In the putative β-barrel portion of the alignment the algorithm predicts eight β-sheets in regions where amino acid conservation is the highest**.** The areas of greatest homology are those of the predicted eight β sheets. A schematic diagram of *B. fragilis* using the amino acid sequence from *B. fragilis* OmpA1 is shown in Figure 4**.** Interestingly, gaps in the alignment of the β-barrel domain fall within loops predicted to be exposed on the outer surface of the bacterium, possibly reflecting different biological function or simple genetic drift.

Proposed model of *B. fragilis* OmpA. Analysis of the predicted 3-dimensional structures based on these sequences was accomplished by threading an alignment made from the 4 *B. fragilis* OmpA sequences, *E.*

coli OmpA and *Pseudomonas* OprF onto the *E. coli* crystal structure using the CONSURF program and visualizing with the Protein Explorer program. The barrel structure is colored according to amino acid conservation. The barrel portion is more conserved than the loops, except for specific patches on some of the loops (Figure 5). Both loop 1 and loop 3 have conserved patches.

DISCUSSION

Publication of the *B. fragilis* genome sequence in 2004 has facilitated studies of *B. fragilis* outer membrane proteins (Cerdeno-Tarraga et al., 2005b;Kuwahara et al., 2004). The *B. fragilis* genome is relatively large (~5.3 x10^6 base pairs vs. 4.2 x10^6 for *E. coli* K12) and there are multiple homologs of many genes, particular membrane protein genes. Significant work has been accomplished on the genes involved in capsule formation, their regulation and importance in immune modulation and host colonization (Comstock & Kasper, 2006;Cassel et al., 2008;Coyne et al., 2008;Liu et al., 2008). Most of the phenotypic and functional description of other *B. fragilis* outer membrane proteins were completed at least a decade before the sequence became available and it is difficult or impossible to identify the proteins described with their respective genes. Odou and colleagues described a 45 kD porin protein in *B. fragilis* (Odou et al., 1998) and later described a complex form of this protein that migrates at ~210 kD when electrophoresed before boiling (Odou et al., 2001). Kanazawa and colleagues isolated three proteins (51K, 92K and 125K proteins) with porin activity (Kanazawa et al., 1995). Unfortunately, neither of the two latter groups identified the proteins by either genetic or proteomic methods. We reported the identification, gene sequence and characterization of *B. fragilis omp200*, a porin gene (Wexler et al., 2002a) and *B. fragilis ompA* (Wexler et al., 2002b) in 2002.

OmpAs in other organisms.

The role of OmpA as a porin molecule that would allow passage of nutrients and/or antibiotics into the cell had been hotly debated (Smith et al., 2007;Nakamura & Mizushima, 1976;Nikaido & Vaara, 1985;Gotoh *et al.*, 1989;Yoshihara & Nakae, 1989). Nikaido proposed that the different channel sizes seen could be explained by different conformations assumed by both OmpA and OprF (Brinkman *et al.*, 2000). Recent understanding is that both the full-length and N-terminal domains of OmpA (and OprF) can form pores of varying sizes (Arora *et al.*, 2000;Brinkman et al., 2000;Brinkman et al., 2000;Sugawara & Nikaido, 1994;Zakharian & Reusch, 2005;Zakharian & Reusch, 2003) and that the larger conformation can, in fact, function as a porin (Smith et al., 2007;Sugawara & Nikaido, 1994;Nikaido *et al.*, 1991). Thus, for *Pseudomonas*, in which OprF is the major porin, the low permeability of the main conformation of OprF

accounts for the low permeability of the OM, and decreased expression of OprF has been implicated in antimicrobial resistance (Pumbwe & Piddock, 2000). In *E. coli*, however, there are other "classical" trimeric porins OmpF and OmpC, that allow passage of solutes and are implicated in antimicrobial resistance (Cohen *et al.*, 1988). Therefore, in *E. coli* the low permeability of the majority of the OmpA molecules does not affect the permeability as significantly (Nikaido, 2001). Other examples of OmpA homologs can be found that are (Zhang *et al.*, 2008) or are not (Bratu *et al.*, 2008) implicated in antimicrobial resistance; whether the OmpA serves as the major porin for the molecule may be a factor (McGowan, Jr., 2006).

We found that *B. fragilis* constructs lacking OmpA1 were smaller in size and less resistant to both SDS and high salt than the parental strain. Similarly, *P. aeruginosa* OprF is important in maintaining structural integrity of the cell and is required for growth at low osmolarity (Woodruff & Hancock, 1989); truncation of the carboxy-terminal domain results in altered cellular morphology (Rawling *et al.*, 1998). In *E. coli* as well, OmpA is implicated in withstanding stresses due to sodium dodecyl sulfate (SDS), acidic environment, and high osmolarity (Wang, 2002). Aside from functioning in bacterial conjugation, phage and colicin receptors (Morona *et al.*, 1984), *E. coli* OmpA is implicated in the invasion of brain microvascular endothelial cells (Huang et al., 2000) and activating human macrophages (Soulas et al., 2000;Wang & Kim, 2002;Wang & Kim, 2002;Prasadarao et al., 1996). The OmpA homologs in *Porphyromonas gingivalis* are being evaluated as possible prophylactic agents for *P. gingivalis*-associated periodontitis (Veith *et al.*, 2001). The role(s) of *B. fragilis* OmpA homologs in these processes have not yet been investigated.

Multiple copies of *ompA* genes in other organisms. *E. coli* OmpA and *Pseudomonas* OprF are the best studied of the OmpA homologs. The *E. coli* K-12 MG1655 genome indicated only one full-length OmpA protein, with multiple shorter membrane proteins and lipoproteins that had the OmpA-like consensus domain, which is important in attachment to the peptidoglycan layer (Ullstrom *et al.*, 1991;Rawling et al., 1998). A search of the *Pseudomonas* PA01 genome with the OprF sequence indicated that there was also only one full length OprF-like protein, while there were multiple shorter proteins (150-~270) that contained the OmpA-like consensus domain. In contrast, two OmpA homologs were found in *Aeromonas salmonicida* and in *Haemophilus ducreyi* (Klesney-Tait *et al.*, 1997;Costello *et al.*, 1996).

Among anaerobes, *ompA* homologs have been studied in two *Porphyromonas* species: *Porphyromonas gingivalis* (Yoshimura et al., 2008;Iwami et al., 2007;Nagano et al., 2005;Imai et al., 2005;Murakami et al., 2002) and *Porphyromonas asaccharolytica* (Magalashvili *et al.*, 2007). In *P. gingivalis*, two OmpA homologs have been described. Studies in *P. gingivalis* have used different strains (ATCC 33277, W83 and W50) and the OmpA homolog "pairs" are variably named: Pgm6/7 (Nagano et al., 2005), 42kDa and 43kD immunoreactive antigens (Nelson *et al.*, 2003), and Omp40/41 (Veith et al., 2001), respectively. The lack of uniform nomenclature somewhat confuses comparisons between them. *P. gingivalis* mutants lacking the OmpA homologs Pgm6/7 had less stable membranes than wild type cells, as evidenced by wavy and irregular outer membrane structure as seen in transmission electron microscopy, and more vesicles were released from cells (Iwami et al., 2007). In *P. asaccharolytica,* an OmpA homolog (Omp-PA) with pore forming ability was isolated from the outer membrane. Further characterization revealed that this porin consists of two different fractions: a heat-modifiable fraction which in its denatured form migrated on SDS-PAGE as a protein with a molecular weight of 41kDa and a heat-resistant fraction which did not change its migration on SDS-PAGE after boiling. A liposome swelling assay revealed that only the heat-resistant fraction was able to transport sugars after its incorporation into the liposomes, although it did not discriminate between differently sized sugars. The authors hypothesized that the heat-modifiable fraction corresponds to the "closed" conformer of Omp-PA, whereas the heat-resistant fraction corresponds to the "open" conformer of the protein (Magalashvili et al., 2007).

Both Pgm 6 and 7 and PG 0694 and 0695 are contiguous genes, respectively, and form single operons. In contrast to the genetic arrangements of the *ompA* homologs in *Porphyromonas,* the *ompA* homologs in *B. fragilis* are not adjacent and do not constitute a single operon. *B. fragilis ompA2* and *ompA4*, the most similar *ompA* homologs, are divergently transcribed and separated by ~1000 bp. Heterodimers have been observed with Omp40/41 in *P. gingivalis* W50 (Veith et al., 2001), and more recently, heterotrimers with Pgm6 and Pgm7 in *P. gingivalis* ATCC 33277 (Nagano et al., 2005). Our earlier analyses with purified *B. fragilis* OmpA1 showed that arabinose could pass through the OmpA1 pore in a liposome assay; also, black lipid bilayer experiments indicated that *B. fragilis* OmpA formed channels of multiple sizes of 0.1–0.3, 0.6 and 0.9

ns (which would be consistent with monomer, dimer and trimer forms). However, we did not find any changes in MICs to any antimicrobials tested, supporting our assertion that *B. fragilis* OmpA1 does not act as a major porin for the organism.

Further investigations of the function of OmpA1 in *B. fragilis* and on the functions of the four surface-exposed loops are underway. Microarray data (submitted for publication) indicates that transcription of many *B. fragilis* outer membrane proteins are affected by the deletion of *ompA1;* thus assignation of function to a single protein might prove difficult. To date, we are not aware of any other bacterium that has four conserved *ompA* homologs (and an additional *ompA* that is somewhat less conserved). Thus far, we are unable to find stress conditions that increase transcription of the other *ompA* homologs and their function in the bacterium remains unclear.

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Table 1: Strains, plasmids and primers used in this study

Strain or plasmid	Description or relevant marker	Source or reference
B. fragilis		
WAL 3501	NCTC 9343 (aka ATCC 25285)	ATCC
WAL 26	*B. fragilis* 638R	
WAL 108	*B. fragilis* ADB77; An isogen of 638R optimised for genetic manipulation , parental strain of deletion mutant. TM400 thyA-, rif^R, $tmp^{R.}$	Baughn & Malamy; [4,5]
WAL 67Ω *ompA1*	*B. fragilis* 638R/pFD516::'*ompA1*''; $erythromycin^R$	This study
WAL 174	*B. fragilis* ADB77/pYT102::*ompA1* updown; $tetracycline^R$	This study
WAL 186 Δ*ompA1*	*ompA* deletant	This study
WAL 360 + *ompA1*	WAL 186 Δ*ompA1* with full length *ompA* reinserted	This study
E. coli		
DH5α	Δ *lacZ* M15	Baughn & Malamy; [4,5]
S17-1	DW1030; host strain for pRK231	Baughn & Malamy; [4,5]
WAL 34	*E. coli* DH5α/pFD516::'*ompa*'; $erythromycin^R$	this study
WAL 169	*E. coli* DH5α/pYT102::*ompA1*updown; $tetracycline^R$	this study
WAL 310	*E coli/pAD242b::up-ompA1-down*	this study
Plasmids		Baughn & Malamy; [4,5]
pFD516		Smith ***
pFD516::'*ompA1*'	pFD516 with 361 bp of internal *ompA* sequence	
pTY102	P15 *ori*, $chloramphenicol^R$, RP4 *oriT*; *B. fragilis* suicide vector, $thyA^+$, $tetracycline^R$	Baughn & Malamy; [4,5]
pTY102::'*ompA1*updown"	pYT102 with 800bp upstream and 800 bp of downstream ompA sequence	
pADB242b	pYT102 derivative, thy defective, tet^R	Baughn & Malamy; [4,5]
pAD242b::up-*ompA1*-down	pADB242b with full length *ompA* and 800 bp of upstream and downstream sequence	
pRK231	RP4 derivative, $tetracycline^R$, ampicillins; broad host –range mobiliser plasmid	Baughn & Malamy; [4,5]

Primers
Restriction endonuclease sites are underlined.

Primer name	**Primer sequence 5' to 3'**	**Reference**
Plasmid specific primers	**ccatcggtgatgtcggc**	
pRY102-forward (61 RAB)	ggcgcgccgtaaggaaagtggctctcag	Baughn & Malamy; [4,5]

pRY102-reverse (1843)	ccatcggtgatgtcggc	Baughn & Malamy; [4,5]
Primers for constructing ompA variant strains		this study
pFD516::'ompA1-internal'F	cagctgattttgtgaagagtgg	this study
pFD516::'ompA1internal'R	cccgggaaacgataagtcaaa	this study
pTY102::'ompA1updown"-UpF	aaggaagcttacagtctgggagtcgctacct	this study
pTY102::'ompA1updown"-UpR	cgttccatggtcccgtaatcgtagtctgctg	this study
pTY102::'ompA1updown"-DownF	ccagccatggtggtggtgtggataaattcg	this study
pTY102::'ompA1updown"-DownR	gcttggatcctcacttaccccatcagtcagg	this study
pAD242b::up-ompA1-down-F	aaggaagcttacagtctgggagtcgctacct	this study
pAD242b::up-ompA1-down-R	gcttggatcctcacttaccccatcagtcagg	this study
Primers for RT PCR		
OmpA1-F	gga tat gac ggt gtt gcc ag	this study
OmpA1-R	tag cag cag cca tgt cat tc	this study
OmpA2-F	Tag aag gtg cat gga cta ct	this study
OmpA2-R	Aac cgc caa tag cat tgg ac	this study
OmpA3-F	act ccg ctg atc aat gtg tc	this study
OmpA3-R	cgt ctg cac gca tag tga ag	this study
OmpA4-F	cca aga tcg acg act atg ct	this study
OmpA4-R	ttc tgg ttc cac ttg gca ct	this study
Primers for qRT PCR analaysis of ompAs1-4		
OmpA1-F-q	cagctgattttgtgaagagtgg	this study
OmpA1-R-q	cccgggaaacgataagtcaaa	this study
OmpA2-F-q	caaccccgacaactttgatt	this study
OmpA2-R-q	taggccttggcaaacgtaag	this study
OmpA3-F-q	tttcatgccgacactttctg	this study
OmpA3-R-q	acggtaacgtgcctggatac	this study
OmpA4-F-q	ggacagcctgccgatactta	this study
OmpA4-R-q	ttaccaaacggagacggaga	this study

Table 2: Quantitative RT PCR of *ompAs 1-4*

	Strain and expression Ct				
gene	**108**	**WAL186Δ*ompA1***	**WAL67Ω*ompA1***	**108/ NaCl**	**186/NaCl**
16s rRNA	22.33	23.17	22.07	21.42	21.16
ompA1	20.21	**0**	22.69	**27.62**	0
ompA2	27.76	27.9	27.25	**36.52**	**38.88**
ompA3	25.44	26.74	25.14	**37.21**	**39.54**
ompA4	30.94	**37.59**	31.89	**0**	**0**

Figure Legends;

Figure 1. Phylogenetic comparisons of *B. fragilis* OmpAs with other members of the OmpA-domain family. Phylogram was based on an alignment using MUSCLE (http://bioinfo.genotoul.fr/multalin/multalin.html) and the tree drawn with Phylodendron (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html).

Figure 2: **SDS-PAGE analysis of OmpA parental, deletant and reinsertant strains.** Figure 2 is a composite of two gels run simultaneously. Lane 1 and Lane 10: Molecular weight (MW) markers 205, 116, 94.7, 66, 4 and 29 kDa, respectively; 2 and 3: *B. fragilis* WAL 108 wild type cell lysates; 4 and 5: WAL 108 wild type Triton pellet (25'C and boiled, respectively); 6 and 7: WAL 186 Δ*ompA1* cell lysates; 8 and 9: WAL 186 Δ *ompA1* Triton pellet (25'C and boiled, respectively); 10: MW markers; 11 and 12: WAL 360 + *ompA1* cell lysates; and 13 and 14: WAL 360 +*ompA*1 Triton pellet (25'C and boiled, respectively).

Figure 3: Effects of oxygen stress on *B. fragilis* (left to right: WAL 638R, WAL108, parental; WAL186Δ*ompA1* (deletant), WAL 360+*ompA1* (reinsertant)

Figure 4. Schematic diagram of the proposed OmpA trans-membrane fold. A two-dimensional representation of the 8-strand □-barrel of *B. fragilis* OmpA1 spanning the outer membrane based on a ClustalW alignment of the sequences of the four *B. fragilis* OmpAs, *E. coli* OmpA, and *P. aeruginosa* OprF. Alignment of the "hinge" region (left panel) with the *E. coli* hinge region was helpful in the sequence prediction. The amino-terminal residue after signal cleavage is Q20 and the carboxy-terminal residue remains K394. □-strands are labeled and are represented by arrows with the first and last predicted amino acid indicated. Loops are indicated by arcs between adjacent □-barrels. Elongated circles represent predicted α helices. The OmpA-domain is located in the periplasm and starts at position V293.

Figure 5. Slab Mode showing *B. fragilis* OmpA barrel threaded onto the *E. coli* OmpA crystal structure.

Upper panel: outside surface of barrel (both sides). Lower panel: Inside surface of barrel (Beta carbons (alpha for Gly) are space-filled. Conservation is color-keyed as indicated (blue=least conserved to maroon=most conserved.)

Reference List

Arora, A., Rinehart, D., Szabo, G. et al. (2000). Refolded outer membrane protein A of *Escherichia coli* forms ion channels with two conductance states in planar lipid bilayers. *J Biol Chem* **275**, 1594-1600.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987). Analysis of Proteins. In *Current protocols in molecular biology.* New York: John Wiley & Sons.

Baughn, A. D. & Malamy, M. H. (2002).A mitochondrial-like aconitase in the bacterium *Bacteroides fragilis*: implications for the evolution of the mitochondrial Krebs cycle.PG -. *Proc Natl Acad Sci U S A* **99**.

Bayley, D. P., Rocha, E. R. & Smith, C. J. (2000). Analysis of *cepA* and other *Bacteroides fragilis* genes reveals a unique promoter structure. *FEMS Microbiol Lett* **193**, 149-154.

Bratu, S., Landman, D., Martin, D. A. et al. (2008). Correlation of antimicrobial resistance with beta-lactamases, the OmpA-like porin, and efflux pumps in clinical isolates of *Acinetobacter baumannii* endemic to New York City. *Antimicrob Agents Chemother* **52**, 2999-3005.

Brinkman, F. S., Bains, M. & Hancock, R. E. (2000). The amino terminus of *Pseudomonas aeruginosa* outer membrane protein OprF forms channels in lipid bilayer membranes: correlation with a three-dimensional model. *J Bacteriol* **182**, 5251-5255.

Cassel, S. L., Sutterwala, F. S. & Flavell, R. A. (2008). The tiny conductor: immune regulation via commensal organisms. *Cell Host Microbe* **3**, 340-341.

Cerdeno-Tarraga, A. M., Patrick, S., Crossman, L. C. et al. (2005a). Extensive DNA inversions in the *B. fragilis* genome control variable gene expression: Supplemental online material. *Science* **307**, 1463-1465.

Cerdeno-Tarraga, A. M., Patrick, S., Crossman, L. C. et al. (2005b). Extensive DNA inversions in the *B. fragilis* genome control variable gene expression. *Science* **307**, 1463-1465.

Chen, R., Schmidmayr, W., Kramer, C. et al. (1980). Primary structure of major outer membrane protein II (OmpA protein) of *Escherichia coli* K-12. *Proc Natl Acad Sci U S A* **77**, 4592-4596.

Cheng, Q., Yu, M. C., Reeves, A. R. et al. (1995). Identification and characterization of a *Bacteroides* gene, *csuF*, which encodes an outer membrane protein that is essential for growth on chondroitin sulfate. *J Bacteriol* **177**, 3721-3727.

Cohen, S. P., McMurry, L. M. & Levy, S. B. (1988).marA locus causes decreased expression of OmpF porin in multiple- antibiotic-resistant (Mar) mutants of *Escherichia coli*. *J Bacteriol* **170**, 5416-5422.

Comstock, L. E., Coyne, M. J., Tzianabos, A. O. et al. (1999). Analysis of a capsular polysaccharide biosynthesis locus of *Bacteroides fragilis. Infect Immun* **67**, 3525-3532.

Comstock, L. E. & Kasper, D. L. (2006). Bacterial glycans: key mediators of diverse host immune responses. *Cell* **126**, 847-850.

Costello, G. M., Vipond, R. & MacIntyre, S. (1996). *Aeromonas salmonicida* possesses two genes encoding homologs of the major outer membrane protein, OmpA. *J Bacteriol* **178**, 1623-1630.

Coyne, M. J., Chatzidaki-Livanis, M., Paoletti, L. C. et al. (2008). Role of glycan synthesis in colonization of the mammalian gut by the bacterial symbiont *Bacteroides fragilis*. *Proc Natl Acad Sci U S A* **105**, 13099-13104.

Finegold, S. M. & Wexler, H. M. (1996). Present studies of therapy for anaerobic infections. *Clin Infect Dis* **23 Suppl 1**, S9-14.

Gallagher, S. (1987). Analysis of Proteins. In *Current Protocols in Molecular Biology*. Edited by Ausubel, F. M. New York: John Wiley and Sons.

Glaser, F., Pupko, T., Paz, I. et al. (2003). ConSurf: identification of functional regions in proteins by surface-mapping of phylogenetic information. *Bioinformatics* **19**, 163-164.

Gotoh, N., Wakebe, H., Yoshihara, E. et al. (1989). Role of protein F in maintaining structural integrity of the *Pseudomonas aeruginosa* outer membrane. *J Bacteriol* **171**, 983-990.

Huang, S. H., Stins, M. F. & Kim, K. S. (2000). Bacterial penetration across the blood-brain barrier during the development of neonatal meningitis. *Microbes Infect* **2**, 1237-1244.

Imai, M., Murakami, Y., Nagano, K. et al. (2005). Major outer membrane proteins from *Porphyromonas gingivalis*: strain variation, distribution, and clinical significance in periradicular lesions. *Eur J Oral Sci* **113**, 391-399.

Iwami, J., Murakami, Y., Nagano, K. et al. (2007). Further evidence that major outer membrane proteins homologous to OmpA in *Porphyromonas gingivalis* stabilize bacterial cells. *Oral Microbiol Immunol* **22**, 356-360.

Kanazawa, K., Kobayashi, Y., Nakano, H. et al. (1995). Identification of three porins in the outer membrane of *Bacteroides fragilis*. *FEMS Microbiol Lett* **127**, 181-186.

Kelley, L. A., MacCallum, R. M. & Sternberg, M. J. (2000). Enhanced genome annotation using structural profiles in the program 3D- PSSM. *J Mol Biol* **299**, 499-520.

Klesney-Tait, J., Hiltke, T. J., Maciver, I. et al. (1997). The major outer membrane protein of *Haemophilus ducreyi* consists of two OmpA homologs. *J Bacteriol* **179**, 1764-1773.

Kuwahara, T., Yamashita, A., Hirakawa, H. et al. (2004). Genomic analysis of *Bacteroides fragilis* reveals extensive DNA inversions regulating cell surface adaptation. *Proc Natl Acad Sci U S A* **101**, 14919-14924.

Laemmli, U. K. & Favre, M. (1973). Maturation of the head of bacteriophage T4. DNA packaging events. *J Mol Biol* **80**, 575-599.

Landau, M., Mayrose, I., Rosenberg, Y. et al. (2005). ConSurf 2005: the projection of evolutionary conservation scores of residues on protein structures. *Nucleic Acids Res* **33**, W299-W302.

Liu, C. H., Lee, S. M., Vanlare, J. M. et al. (2008). Regulation of surface architecture by symbiotic bacteria mediates host colonization. *Proc Natl Acad Sci U S A* **105**, 3951-3956.

Magalashvili, L., Pechatnikov, I., Wexler, H. M. et al. (2007). Isolation and characterization of the Omp-PA porin from *Porphyromonas asaccharolytica*, determination of the omp-PA gene sequence and prediction of Omp-PA protein structure. *Anaerobe*.

McGowan, J. E., Jr. (2006). Resistance in nonfermenting gram-negative bacteria: multidrug resistance to the maximum. *Am J Infect Control* **34**, S29-S37.

Mineoka, T., Awano, S., Rikimaru, T. et al. (2008). Site-specific development of periodontal disease is associated with increased levels of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* in subgingival plaque. *J Periodontol* **79**, 670-676.

Morona, R., Klose, M. & Henning, U. (1984).*Escherichia coli* K-12 outer membrane protein (OmpA) as a bacteriophage receptor: analysis of mutant genes expressing altered proteins. *J Bacteriol* **159**, 570-578.

Murakami, Y., Imai, M., Nakamura, H. et al. (2002). Separation of the outer membrane and identification of major outer membrane proteins from *Porphyromonas gingivalis*. *Eur J Oral Sci* **110**, 157-162.

Nagano, K., Read, E. K., Murakami, Y. et al. (2005). Trimeric structure of major outer membrane proteins homologous to OmpA in *Porphyromonas gingivalis*. *J Bacteriol* **187**, 902-911.

Nakamura, H. & Mizushima, S. (1976). Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *Escherichia coli* K12. *J Biochem* **80**, 1411-1422.

Nelson, K. E., Fleischmann, R. D., DeBoy, R. T. et al. (2003). Complete genome sequence of the oral pathogenic Bacterium *Porphyromonas gingivalis* strain W83. *J Bacteriol* **185**, 5591-5601.

Nielsen, H., Engelbrecht, J., Brunak, S. et al. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* **10**, 1-6.

Nikaido, H. (2001). Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Semin Cell Dev Biol* **12**, 215-223.

Nikaido, H., Nikaido, K. & Harayama, S. (1991). Identification and characterization of porins in *Pseudomonas aeruginosa*. *J Biol Chem* **266**, 770-779.

Nikaido, H. & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49**, 1-32.

Odou, M. F., Singer, E. & Dubreuil, L. (2001). Description of complex forms of a porin in *Bacteroides fragilis* and possible implication of this protein in antibiotic resistance. *Anaerobe* **7**, 219-225.

Odou, M. F., Singer, E., Romond, M. B. et al. (1998). Isolation and characterization of a porin-like protein of 45 kilodaltons from *Bacteroides fragilis*. *FEMS Microbiol Lett* **166**, 347-354.

Prasadarao, N. V., Wass, C. A., Weiser, J. N. et al. (1996). Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infect Immun* **64**, 146-153.

Pumbwe, L. & Piddock, L. J. (2000). Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **44**, 2861-2864.

Pumbwe, L., Ueda, O., Chang, A. et al. (2005).*Bacteroides fragilis* BmeABC Efflux transporters are coordinately expressed and additively confer intrinsic multi-substrate resistance. *Abstracts of the 2005 Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, D C*.

Pumbwe, L., Ueda, O., Yoshimura, F. et al. (2006).*Bacteroides fragilis* BmeABC Efflux Systems Additively Confer Intrinsic Antimicrobial Resistance. *J Antimicrob Chemother* **58**, 37-46.

Rawling, E. G., Brinkman, F. S. & Hancock, R. E. (1998). Roles of the carboxy-terminal half of *Pseudomonas aeruginosa* major outer membrane protein OprF in cell shape, growth in low-osmolarity medium, and peptidoglycan association. *J Bacteriol* **180**, 3556-3562.

Reeves, A. R., D'Elia, J. N., Frias, J. et al. (1996). A *Bacteroides thetaiotaomicron* outer membrane protein that is essential for utilization of maltooligosaccharides and starch. *J Bacteriol* **178**, 823-830.

Reeves, A. R., Wang, G. R. & Salyers, A. A. (1997). Characterization of four outer membrane proteins that play a role in utilization of starch by *Bacteroides thetaiotaomicron*. *J Bacteriol* **179**, 643-649.

Rocha, E. R., Tzianabos, A. O. & Smith, C. J. (2007). Thioredoxin reductase is essential for thiol/disulfide redox control and oxidative stress survival of the anaerobe *Bacteroides fragilis*. *J Bacteriol* **189**, 8015-8023.

Schirmer, T. & Cowan, S. W. (1993). Prediction of membrane-spanning beta-strands and its application to maltoporin. *Protein Science* **2**, 1361-1363.

Shipman, J. A., Berleman, J. E. & Salyers, A. A. (2000). Characterization of four outer membrane proteins involved in binding starch to the cell surface of *Bacteroides thetaiotaomicron*. *J Bacteriol* **182**, 5365-5372.

Smith, S. G., Mahon, V., Lambert, M. A. et al. (2007).A molecular Swiss army knife: OmpA structure, function and expression. *FEMS Microbiol Lett* **273**, 1-11.

Soulas, C., Baussant, T., Aubry, J. P. et al. (2000). Outer membrane protein A (OmpA) binds to and activates human macrophages. *J Immunol* **165**, 2335-2340.

Stintzi, A., Marlow, D., Palyada, K. et al. (2005). Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. *Infect Immun* **73**, 1797-1810.

Struyve, M., Moons, M. & Tommassen, J. (1991). Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. *J Mol Biol* **218**, 141-148.

Sugawara, E. & Nikaido, H. (1994). OmpA protein of *Escherichia coli* outer membrane occurs in open and closed channel forms. *J Biol Chem* **269**, 17981-17987.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-4680.

Ullstrom, C. A., Siehnel, R., Woodruff, W. et al. (1991). Conservation of the gene for outer membrane protein OprF in the family *Pseudomonadaceae*: sequence of the *Pseudomonas syringae oprF* gene. *J Bacteriol* **173**, 768-775.

Veith, P. D., Talbo, G. H., Slakeski, N. et al. (2001). Identification of a novel heterodimeric outer membrane protein of *Porphyromonas gingivalis* by two-dimensional gel electrophoresis and peptide mass fingerprinting. *Eur J Biochem* **268**, 4748-4757.

Vogel, H. & Jahnig, F. (1986). Models for the structure of outer-membrane proteins of *Escherichia coli* derived from raman spectroscopy and prediction methods. *J Mol Biol* **190**, 191-199.

von Heijne, G. (1985). Signal sequences. The limits of variation. *J Mol Biol* **184**, 99-105.

von Heijne, G. (1983). Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem* **133**, 17-21.

Wang, Y. (2002). The function of OmpA in *Escherichia coli*. *Biochem Biophys Res Commun* **292**, 396-401.

Wang, Y. & Kim, K. S. (2002). Role of OmpA and IbeB in *Escherichia coli* K1 Invasion of Brain Microvascular Endothelial Cells In Vitro and In Vivo. *Pediatr Res* **51**, 559-563.

Wexler, H. M. (1991). Susceptibility testing of anaerobic bacteria: myth, magic, or method? *Clin Microbiol Rev* **4**, 470-484.

Wexler, H. M., Molitoris, E., Murray, P. R. et al. (1996). Comparison of spiral gradient endpoint and agar dilution methods for susceptibility testing of anaerobic bacteria: a multilaboratory collaborative evaluation. *J Clin Microbiol* **34**, 170-174.

Wexler, H. M., Read, E. K. & Tomzynski, T. J. (2002a). Characterization of *omp200*, a porin gene complex from *Bacteroides fragilis*: *omp121* and *omp71*, gene sequence, deduced amino acid sequence and predictions of porin structure. *Gene* **283**, 95-105.

Wexler, H. M., Read, E. K. & Tomzynski, T. J. (2002b). Identification of an OmpA protein from *Bacteroides fragilis*: *ompA* gene sequence, OmpA amino acid sequence and predictions of protein structure. *Anaerobe* **8**, 180-191.

Wong, C., Sridhara, S., Bardwell, J. et al. (2000). Heating greatly speeds Coomasie Blue staining and destaining. *BioTechniques* **28**, 426-432.

Woodruff, W. A. & Hancock, R. E. (1989).*Pseudomonas aeruginosa* outer membrane protein F: structural role and relationship to the *Escherichia coli* OmpA protein. *J Bacteriol* **171**, 3304-3309.

Yoshihara, E. & Nakae, T. (1989). Identification of porins in the outer membrane of *Pseudomonas aeruginosa* that form small diffusion pores. *J Biol Chem* **264**, 6297-6301.

Yoshimura, F., Murakami, Y., Nishikawa, K. et al. (2008). Surface components of *Porphyromonas gingivalis*. *J Periodontal Res*.

Zakharian, E. & Reusch, R. N. (2005). Kinetics of folding of *Escherichia coli* OmpA from narrow to large pore conformation in a planar bilayer. *Biochemistry* **44**, 6701-6707.

Zakharian, E. & Reusch, R. N. (2003). Outer membrane protein A of *Escherichia coli* forms temperature-sensitive channels in planar lipid bilayers. *FEBS Lett* **555**, 229-235.

Zhang, R., Yang, L., Cai, J. C. et al. (2008). High-level carbapenem resistance in a *Citrobacter freundii* clinical isolate is due to a combination of KPC-2 production and decreased porin expression. *J Med Microbiol* **57**, 332-337.